MINIREVIEW

Historical Data Analyses and Scientific Knowledge Suggest Complete Removal of the Abnormal Toxicity Test as a Quality Control Test

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ABSTRACT: In the early 1900s, the abnormal toxicity test (ATT) was developed as an auxiliary means to ensure safe and consistent antiserum production. Today, the ATT is utilized as a quality control (QC) release test according to pharmacopoeial or other regulatory requirements. The study design has not been changed since around 1940. The evidence of abnormal toxicity testing as a prediction for harmful batches is highly questionable and lacks a scientific rationale. Numerous reviews of historical ATT results have revealed that no reliable conclusions can be drawn from this QC measure. Modern pharmaceutical manufacturers have thorough control of the manufacturing process and comply with good manufacturing practice rules. Contaminants are appropriately controlled by complying with the validated manufacturing processes and strict QC batch release confirming batch-to-batch consistency. Recognizing that product safety, efficacy, and stability can be ensured with strict QC measures, nowadays most regulatory authorities do not require the ATT for most product classes. In line with the replacement, reduction, and refinement (3Rs) initiative, the test requirement has been deleted from approximately 80 monographs of the European Pharmacopoeia and for the majority of product classes in the United States. For these reasons, it is recommended that the ATT should be consistently omitted world-wide and be removed from pharmacopoeias and other regulatory requirements. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:3349–3355, 2014
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INTRODUCTION

The abnormal toxicity test (ATT) [European Pharmacopoeia (EP) nomenclature][1] is also referred to as the general safety (US reference)[2] or innocuity test (WHO nomenclature).[3] The principle of this animal test consists of a single injection of a specified volume of a product batch into guinea pigs and/or mice

followed by an observation period. Typically, a batch passes the test if the findings seen in animals follow the below criteria:

1. animals survive the test period;
2. animals do not exhibit any response, which is not specific for or expected from the product and may indicate a difference in its quality; and
3. animals weigh not less at the end of the test period than that at the time of injection.[2]

The ATT was developed in the early 1900s, when production processes and quality control (QC) for biological products were poorly established and licensing procedures did not yet exist. At this time, the test was intended to ensure the safe and consistent production of serum products, for example, to titrate the preservative phenol level of diphtheria antiserum. Analytical techniques were not available to appropriately detect phenol in serum products. Therefore, mice—as a susceptible

species—were used for the detection of potentially toxic phenol levels. The test with guinea pigs was introduced around 1900 as a biological indicator for the presence of tetanus toxin in antiserum preparations.[4,5]

The test was later expanded to a general safety test to detect extraneous contaminants (other than, for example, bacterial endotoxins) in biological products. In spite of significant evolution of analytical techniques as well as advanced process understanding and validation approaches, this biological test remains and changed from being an analytical test to an additional safety test intended to detect product/process contaminants to avoid batch-to-batch differences in quality.

The test has not significantly changed since around 1940. National implementation leads to today's variations between the different pharmacopoeias/international requirements. Table 1 presents requirements from the pharmacopoeias of Europe,[1] the United States,[2] Russia,[6] and China[7] as well as WHO requirements.[3]

Of note, the administration volumes in the case of intravenous (i.v.) dosing do not comply with today's best practices and animal welfare considerations. According to Diehl et al.,[8] a maximum of 5 mL/kg should be administered to mice, which would result in a maximum volume of 0.1 mL for mice with a body weight of around 20 g. Following the pharmacopoeias/requirements (0.5–1.0 mL administration volume), a mouse receives 5–10-fold of the volume considered good practice.

HISTORICAL DATA ANALYSES

Publications by the German Paul Ehrlich Institute provide evidence that the test does not serve its purpose and does not add any further information to that already obtained from QC release testing under good manufacturing practice (GMP).[9] A retrospective analysis of several thousand test results conducted for vaccines revealed that there were no true positive results[10,11] and no batch rejection was obtained by the authority.[12] In conclusion, there is no evidence that the ATT is useful as a predictor or control for harmful batches. Table 2 presents details on the analysis of historical data from Kraemer et al.[12]

As a result of the historical experience, the US Food and Drug Administration (FDA) amended the respective regulations regarding general biological products standards by adding an administrative procedure for obtaining exemptions from the test requirements.[13] A similar conclusion was drawn by the European agency, and the test was removed from over 80 product monographs.[11,14] For details, see the section given below.

TEST PERFORMANCE

Specificity (False Positive Results)

The ATT is nonspecific, as many factors other than contaminants can influence the result (e.g., body weight, species and strain differences, stress levels of the animals). Accordingly, misinterpretation of responses caused by the active ingredients themselves, or formulation components, may lead to false positive results, for example, as administered concentrations may be unrealistically high compared with what is administered to humans.[15] The formulations are optimized for safe use

in humans but not in mice or guinea pigs at such high concentrations.

Depending on the test design, a fixed volume is administered, irrespective of the dose used in humans. Thus, the full human dose may be administered to guinea pigs of 250–400 g body weight.[1] In this case, assuming a human body weight of 60 kg, a guinea pig would receive 150-fold of the human dose. A mouse of 20 g would receive 3000-fold of the human dose.

As already mentioned, formulation components (e.g., preservative and vaccine adjuvants) may cause false positive responses. There are some examples of drug products that have yielded false positive test results:

- A response following intraperitoneal injection of high concentrations of benzyl alcohol, which is used as a formulation component for a recombinant protein.
- A reaction caused by the high sugar content in an oral pediatric vaccine, when administered according to a national pharmacopoeia by i.v. injection.

Reproducibility

Kraemer et al.[12] investigated the test reproducibility if the ATT is performed with strict adherence to the same study design/protocol (German Pharmacopoeia DAB 10). Identical batches tested in different laboratories have produced significantly different test results. Positive results never showed a correlation with product quality or contamination, and the same batches passed the ATT in subsequent test repetitions.

In addition, the ATT design is not harmonized and varies between national pharmacopoeias/international requirements (Table 1).

We conclude that test responses of the same batch may be unpredictable and may give contradictory results if performed:

- repeatedly in the same laboratory,
- in different laboratories according to the same protocol, and
- in different countries according to different protocols.

Reliability

Reliability is a measure of consistency/reproducibility. Considering the lack of reproducibility, the ATT must be classified as unreliable.

Suitability

For common analytical procedures (e.g., quantitative tests for impurities' content), it is mandatory to demonstrate suitability for the intended purpose. Typical parameters of such validation are precision (repeatability) and specificity.[16] The ATT is neither reproducible nor specific. Accordingly, the ATT would not fulfill international validation criteria of analytical methods.

No adequate positive control is possible as the test seeks for unknown contamination and nonspecific toxicity. Furthermore, the ATT lacks explicit acceptance criteria, as no definite endpoint is defined ("signs of ill health,"[1] "significant signs of toxicity,"[3] and "abnormal reaction"[7]).

In conclusion, this animal test is considered as not suitable for its intended purpose.

Table 1. Comparison of Test Conditions According to Different Pharmacopoeias/Requirements (Examples)

Scope	European Pharmacopoeia[1] General Test	European Pharmacopoeia[1] Immunosera/Vaccines	United States[a] Biological Products (with exemptions[b])	WHO[3] Vaccines	Russian Pharmacopoeia[6] General Test	Russian Pharmacopoeia[6] Vaccines/Sera	Chinese Pharmacopoeia[7] Biologics/Vaccines	Chinese Pharmacopoeia[7] Chemicals, Traditional Medicines
Blank control	No	No	No	No	No	No	Blank control	No
Animal quantity	5 mice	5 mice	≥5 mice	5 mice	5 mice	5 mice	5 mice	5 mice
Body weight (g)	17–24	2 guinea pigs 17–24 (m)	≥2 guinea pigs <22 (m)	2 guinea pigs 17–22 (m)	19–21	2 guinea pigs 17–20 (m)	2 guinea pigs 18–22 (m)	17–20
Dose/administration volume	One human dose; ≤1.0 mL	250–400 (gp) One human dose; ≤1.0 mL (m) ≤5.0 mL (gp)	<400 (gp) ≤0.5 mL (m); ≤5.0 mL (gp)	250–350 (gp) One human dose; ≤1.0 mL (m) ≤1.0 mL (gp)	0.5 mL	250–300 (gp) One human dose; ≤1.0 mL (m) ≤5.0 mL (gp)	250–350 (gp) 0.5 mL (m); 5.0 mL (gp)	0.5 mL (m)
Injection route	i.v.	i.p.	i.p. or following the approved route of product administration	i.p.	i.v.	i.p.	i.p.	Following the approved route of product administration
Observation time	24 h	7 days	48 h	48 h	48 h	7 days	7 days	2 days
Acceptance criteria	No animal dies within 24 h or within such time as specified in the individual monograph	No animal shows signs of ill health	No animal dies or exhibits any response, which is not specific for or expected from the product and may indicate a difference in its quality. No loss of body weight	No animal dies within at least 7 days or shows significant signs of toxicity	No animal dies within the specified follow-up period	No animal dies within at least 7 days, shows significant signs of toxicity, or a decrease in body weight	All animals remain healthy and survive the observation period, without any abnormal reaction, and with an increase in body weight by the end of observation period	All animals survive the observation period
Retest(s) number/description	One. If one animal dies, repeat the test	One. If one animal dies or shows signs of ill health, repeat the test	Two. If the initial test/first repeat test fails, a repeat test may be conducted	No	One. If an animal dies, repeat the experiment with five mice (20 ± 0.5 g)	One. If an animal dies, shows clinical signs of intoxication or a decrease in body weight, repeat experiment under the same conditions	One. If the test fails, it may be repeated once with 10 mice/4 guinea pigs	One. If the test fails, it may be repeated once with 10 mice (18–19 g)

[a]The United States Pharmacopoeia (USP 36) refers to US Code of Federal Regulations (21 CFR, Part 610).[2]
[b]Exemptions: therapeutic DNA plasmid products, therapeutic synthetic peptide products of 40 or fewer amino acids, monoclonal antibody products for *in vivo* use, or therapeutic recombinant DNA-derived products.
i.p., intraperitoneal; i.v., intravenous; m, mice; gp, guinea pigs.

Number of Tests	Analyzed Preparations	Used Mice	Used Guinea Pigs	Batch Rejections
5896	416	30193	12420	0

Would Modification Lead to an Improved Test Performance?

Mizukami et al.[17] made an effort to improve the ATT design for vaccines (Fig. 1). It is based on the observation that body weight changes of animals are characteristic for each vaccine, and such standardized changes can be used as references for evaluating vaccines. In addition to body weight analysis, histopathological and hematological analyses are proposed in case of required retests.

In our opinion, this modified scheme does not overcome the principal deficiencies of the test as discussed above, or the inherent variations of the animal model. This variability is also evident in the fact that still up to two retests are foreseen in the scheme. Furthermore, the test design is not considered appropriate to be used as a routine QC release test due to the high experimental efforts (statistical and pathological/hematological analysis) and the long test duration (several weeks including retests). From an ethical perspective, such studies are not in line with animal welfare considerations as a significant number of animals are used for the initial statistical analysis (in order to standardize body weight changes after inoculation of various vaccines) and the potential retests.

CONTROL OF CONTAMINANTS

Scientifically, there is no rationale as to why an animal test for batch release would be more appropriate than other measures to detect and control contamination. For such a test to be adequate and useful, a clear understanding of the mechanism

of how contaminations lead to the study read-out, i.e. positive result, should be in place.

Furthermore, it should be known based on the understanding of the manufacturing process what potential contaminants might be and of what nature they could be. The endpoints and acceptance criteria should be deduced from this understanding so that any QC test can be appropriately validated.

Modern methodologies, such as those presented in Table 3, are far better suited and have a clear scientific rationale because the relationship between the measured endpoint and the causing contamination (e.g., bacterial endotoxin) is well understood and established.

A set of measures are nowadays available to detect and control different types of contaminants. These include:

- extended product characterization during process development and process validation,
- manufacture according to GMPs, and
- routine QC release testing, which verifies batch-to-batch consistency and ensures that a specific batch has been manufactured according to the previously validated process.

Table 3 lists the current control measures used to verify the absence of certain contaminants in a product batch, exemplified by parenteral preparations.[11,14]

MODERN PRODUCT DEVELOPMENT ENSURES COMPREHENSIVE PROCESS UNDERSTANDING AND WELL-CHARACTERIZED PRODUCTS

Nowadays, pharmaceutical manufacturing is highly regulated and controlled. The modern pharmaceutical industry together with regulatory authorities have established appropriate control of the manufacturing process through substantially advanced process understanding, in-process controls, validation
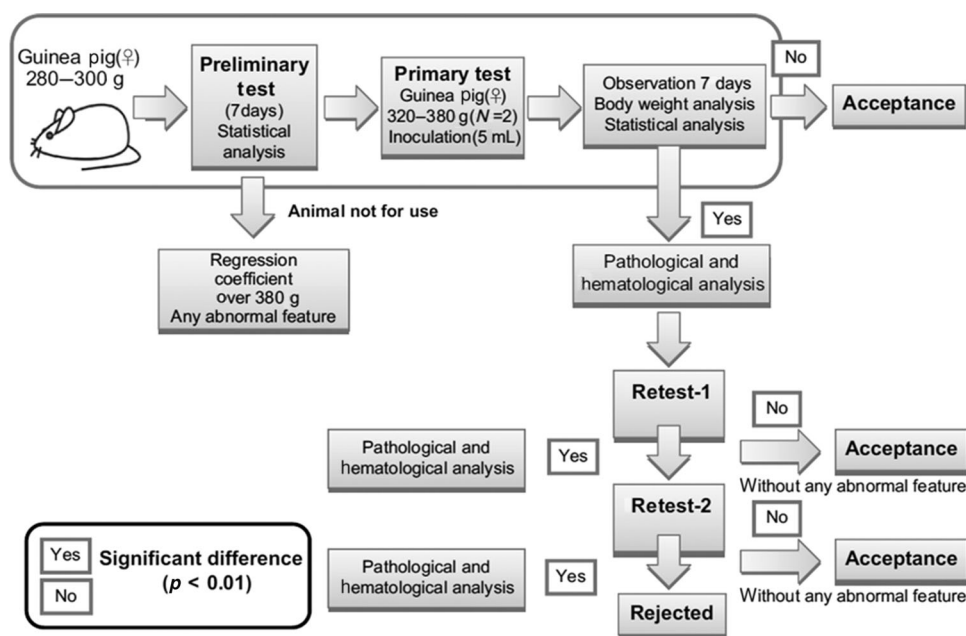

Figure 1. Proposed test scheme according to Mizukami et al.[17]

Table 3. Measures to Verify the Absence of Different Types of Contaminants (Examples)

Type of Contaminant	Measure to Verify the Absence of Contaminants in a Product Batch
Microbiological	– Bioburden test (in-process control) – Sterility test
Pyrogen[a]	– Validation of depyrogenization (as part of the process validation)
Endotoxin	– Bacterial endotoxins (limulus amebocyte lysate) test
Residual contaminants[b]	– Extended product characterization – Process validation – Manufacture under GMP – QC during batch release to confirm batch-to-batch consistency

[a]The formerly used rabbit pyrogens test has been replaced by the generally accepted bacterial endotoxins test in numerous EP monographs.[11]

[b]The formerly used abnormal toxicity test has been deleted based on historical review in numerous EP monographs.[11,14]

of the manufacturing process, and release testing complying with international GMP standards.

During formulation and process development, many studies are conducted with different formulation components (including preservatives) to investigate degradation profiles, product compatibility with various materials/surfaces, and leachables, which may be sources for contaminants.[18–21] Pharmaceutical compounds are tested extensively with regard to their safety/toxicity profile in *in vitro* assays and animals models as well as in clinical trials in accordance with international (e.g., International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) and national guidelines. A marketing authorization is granted by the relevant health authorities only when a positive benefit/risk assessment can be demonstrated.

Today, pharmaceutical manufacturers produce highly developed medicines with well-defined purity and safety characterisics. Risk of contamination is extremely low if a manufacturer complies with GMP rules (e.g., globally recognized regulations[22–25]) and if consistency in production is guaranteed.[26,27] Abnormal product contamination is extremely unlikely if the validated manufacturing process is followed.

Appropriate analytical methods (e.g., mass spectrometry applications) are capable of detecting contamination and ensure batch-to-batch consistency. Advanced product testing is applied for the extended product characterization and release testing.

RELEASE SPECIFICATIONS ARE SET ACCORDING TO INTERNATIONAL REQUIREMENTS AND ENSURE PRODUCT SAFETY, EFFICACY, AND STABILITY

Many multinational manufacturers supply innovative medicines globally. Thus, a batch is usually released for use in the global market. Accordingly, all countries get the same high quality drug. In line with international regulations, in most instances abnormal toxicity testing is not part of the release specifications for these globally marketed products. For example, European Medicines Agency (EMA)- and FDA-approved specifications for commercial drug products do not require abnormal toxicity testing as part of the QC release analysis

for the majority of product classes. However, a batch already released for EU and/or the United States would have to be tested for abnormal toxicity in other countries, for example, the Russian Federation[6] and China,[7] to be released for the local market. To the best of our knowledge, no batch which met the EMA- or FDA-approved specifications delivered a positive ATT result in both of these countries (apart from false positive test results, as aforementioned).[10–12]

INCREASING INTERNATIONAL CONVERGENCE

Several health authorities have conducted an evaluation of the ATT and came to the same conclusion.

European Pharmacopoeia generally does not require abnormal toxicity testing in the monographs for "parenteral preparations,"[28] "monoclonal antibodies for human use,"[29] or "products of recombinant DNA technology."[30] Aforementioned reviews of test results revealed that no additional value could be concluded from abnormal toxicity testing. As a consequence, and in accordance with the European Convention on the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, the test has been deleted from approximately 80 monographs for biotechnological products, blood products, antibiotics, and vaccines based on the review of historical data.[11,14] In addition, further replacement alternatives for abnormal toxicity testing for batch release of existing vaccines have been currently evaluated and strongly recommended.[10,31–35]

The US Code of Federal Regulations Title 21 requires general safety testing be done for biological products (21 CFR, Part 610.11[2]). However, FDA realized that "after more than a decade of experience with these products, we found that we could evaluate many aspects of a biological product's safety, purity, or potency with tests other than those prescribed in part 610."[13] Thus, the FDA amended the biologics regulations regarding general biological products standards by adding an administrative procedure for obtaining exemptions from the general safety test requirements[13]: 21 CFR, Part 601.2 (Ref. 36) specifies that the test is exempted as a requirement for license applications for therapeutic DNA plasmid products, therapeutic synthetic peptide products of 40 or fewer amino acids, monoclonal antibody products for *in vivo* use, or therapeutic recombinant DNA-derived products.

In 2002, the WHO Expert Committee on Biological Harmonization "noted that, in one region of the world, the abnormal toxicity test had been deleted for most products. This was linked to the implementation of, and compliance with, good manufacturing practices and, where this occurred, there was abundant evidence that the abnormal toxicity test did not provide additional assurances of the quality of the product."[37]

ANIMAL WELFARE

The EU adopted a new directive on the protection of animals used for scientific purposes (2010/63/EU[38]). The directive plays a significant role in minimizing the number of animals used in experiments and the European Directorate for the Quality of Medicines & HealthCare continues to push forward the implementation of replacement, reduction, and refinement (3Rs) alternatives.[39]

The substantial number of laboratory animals used for the ATT cannot be justified in view of its unproven and questionable suitability to detect contaminants and increase the product safety.[15] As a consequence, the *European Convention on the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes* reviewed the test for 3Rs. As aforementioned, the test has been consequently deleted from numerous EP monographs.[11,14]

CONCLUSIONS

The ATT was implemented in the early 1900s as auxiliary means for production, i.e., to titrate the level of the preservative phenol in antiserum preparations, and has persisted for the detection of contaminants, without clear rationale and reasoning. The test lacks scientific merit and is neither specific, reproducible, reliable, nor suitable for the intended use. Considering its performance, the ATT is regarded as not appropriate as a QC release test, which should be capable of enabling batch release decisions based on explicit and reproducible results.

A retrospective analysis of several thousand test results revealed that no true positive result and no batch rejection were obtained. Positive results never showed a correlation to the product quality or contamination. Accordingly, the test does not provide added value for QC. This conclusion was also drawn by the FDA considering that safety, purity, and potency of products are ensured without the ATT.

The ATT has therefore been deleted from numerous product monographs, and test exemptions are granted for many product classes, respectively. However, the test is still required as a safety test according to a number of pharmacopoeias and other regulatory requirements for certain product classes.

Based on the rationale provided in this review and in line with the scientific knowledge and regulatory trends outlined herein, it is considered fully justified to completely eliminate abnormal toxicity testing from pharmacopoeias and other regulatory requirements as already recommended by various scientific experts.[12,15,35,40,41] This would also be in agreement with animal welfare concerns (e.g., 3Rs initiatives) and contemporary directives on the protection of animals.

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