20 Critical Questions

for Ensuring Inhaled Chemical and Agrochemical Registration

labcorp

Abbreviations

APS: Aerodynamic particle sizer

BALF: Bronchoalveolar lavage fluid

DART: Developmental and reproductive toxicology

ECHA: European Chemicals Agency

EOGRTS: Extended one-generation reproductive toxicity study

EPA: Environmental Protection Agency

GHS: Globally harmonized system of classification and labeling of chemicals

GLP: Good laboratory practice

GSM: Geometric standard deviation

JMAFF: Japanese Ministry of Agriculture, Forestry and Fisheries

JMETI: Japanese Ministry of Economy, Trade and Industry

JMHLW: Japanese Ministry of Health, Labour and Welfare

MMAD: Mass median aerodynamic diameter

OCSPP: Office of Chemical Safety and Pollution Prevention

OECD: Organisation for Economic Co-operation and Development

OPPTS: Office of Prevention, Pesticides and Toxic Substances (U.S. Environmental Protection Agency)

PTFE: Polytetrafluoroethylene

QA: Quality assurance

REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals

Contents

Introduction

Inhalation studies are mandatory in many regulatory situations where it is critical to understand the potential risk posed by respiratory exposure to a chemical or agrochemical.

Results from inhalation studies inform substance approval and downstream risk assessment and risk management planning, so getting them right impacts both access to a market as well as how a substance is used in practice. One of the first challenges is establishing how feasible and appropriate an inhalation study actually is, based on the physicochemistry of a substance and any known toxicity information.

The studies themselves are complex and challenging, as delivery of test articles via the inhalation route requires careful attention to substance aerolization and particle size as well as constant control and monitoring of the inhaled atmosphere over a prolonged time to ensure correct dosing. Add to that complex animal welfare considerations that must guarantee welfare-compliant delivery systems for individual animals or family groups, which are often necessary for DART studies, like EOGRTS.

All of these considerations impact the potential success of inhalation studies, so as you prepare for the preliminary characterization trials required by regulators, read our answers to 20 frequently asked questions. These will empower you to perform those trials and then set up and monitor an inhalation toxicology study with confidence.

Logistics

1. How quickly can you start an inhalation study?

Standard regulatory designs for toxicity studies are set out in guidelines from the OECD and, in the U.S., the U.S. EPA OCSPP (formerly Office of Prevention, Pesticides and Toxic Substances or OPPTS). Toxicity studies are one of the requirements for registration of chemical substances. For example, in Europe, this means registering with the ECHA under REACH guidelines, and in Korea registering under K-REACH or the Chinese Ministry of Ecology and Environment in China.

Inhalation studies that follow standard regulatory designs can generally be started as quickly as a standard toxicology study by any other administration route, providing the test article is available and the appropriate paperwork is complete (study protocol, certificate of analysis with purity, expiry dates and batch number). However, you will need to consider whether the study has special design considerations or the test article has unusual physicochemical properties, e.g., whether there are unusual endpoints or the test article is a nanomaterial. In such instances, Labcorp can help you with the discussions you will need to have with regulatory authorities in advance of the study start.

Thought must also be given to the need for preliminary characterization trials to establish appropriate aerosol generation and exposure systems.

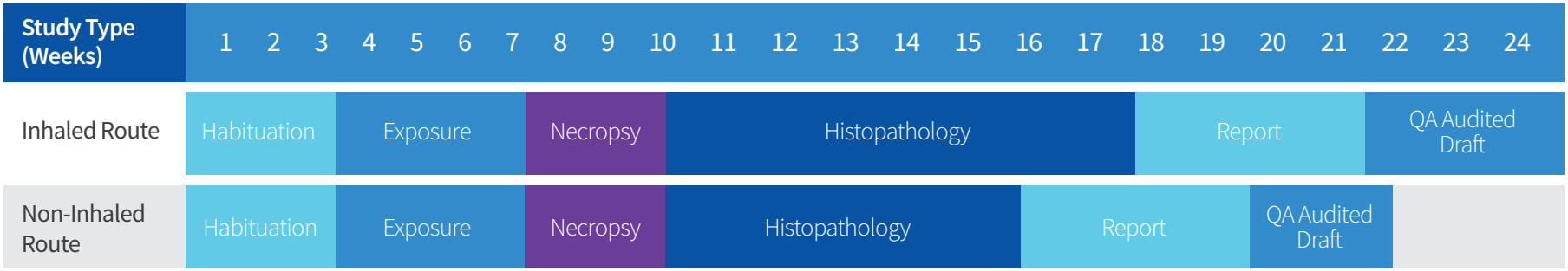
Preliminary characterization must be conducted in advance of starting the animal study and reporting on this is an expectation in guidelines (e.g., see paragraph 47 in OECD 4331). Given sufficient planning, this work rarely impacts the study scheduling. However, analytical determination of concentration and particle size is needed in support of an inhalation study or program, and this must be completed in advance of characterization trials.

See Questions 17–20 for more on preliminary characterization trials

2. Why are reporting timelines different for inhalation studies?

All oral, dermal and inhalation repeat-dose studies require histopathology. However, studies by the inhalation route are more time consuming as the histopathology assessment is more complex. Detailed histopathology of the respiratory tract is a necessary component of studies by the inhalation route and there is separate guidance for it (OECD 1252). This includes processing and examination of the nasal turbinates, which requires a preliminary decalcification step to dissolve the surrounding bone structures. Depending on species and number of animals, this adds 1–2 weeks to the reporting timeline, compared with an oral or dermal study.

Figure 1: Timelines for theoretical 28-day exposure studies by inhaled and non-inhaled routes.



Another component required in studies by the inhalation route, though not impacting on the overall reporting timeline, is the aerosol technology expert report. This describes the exposure system, the prestudy characterization work, the achieved exposure levels and achieved particle-size distribution as described in OECD GD39.[3] This document is the principal guidance on regulatory expectations relating to technical aspects of the various OECD inhalation studies.

3. If performing a '6 pack' of studies for GHS registration, why is the inhalation acute study always conducted last?

Oral and dermal toxicity studies are required in advance of starting an inhalation study because they assess corrosivity and irritancy. The results can then be used to confirm that the inhalation study is still appropriate and warranted.

Guidelines state inhalation testing at corrosive or irritating concentrations should be avoided unless there are specific regulatory needs such as emergency planning.[4]

There is specific guidance on this from several different regulatory sources.

In Health Effects Test Guidelines (August 1998) OPPTS 870.1300 - Acute Inhalation Toxicity[5] [Section (e) Conventional acute toxicity test], the U.S. EPA and OPPTS state:

"...dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out."

This is endorsed by Labcorp on animal welfare grounds and is further supported by OECD 433: Guidelines for the Testing of Chemicals:[1]

"concentrations that are expected to cause marked pain and distress, due to corrosive or severely irritant actions, should not be administered"

Labcorp does not allow conventional acute testing by the inhalation route for substances known to be corrosive or highly irritant, on animal welfare grounds. Instead, conducting a repeat-dose study using noncorrosive concentrations is advised.[6]

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4. Why are inhalation studies so much more expensive than oral and dermal studies?

Inhalation studies are more expensive for several reasons. The main reasons are given in Table 1. These demand a very high level of specialized technical expertise, which takes considerable investment in training and in dedicated aerosol technology and technical groups.

Table 1: Factors raising the cost of inhalation toxicity studies, relative to oral and dermal studies.

Legislation	• Animal dosing is a licensed procedure covered by legislation, e.g., the Animals (Scientific Procedures) Act 1986 in the UK;[7] therefore, staff need to be present for the whole dosing duration, which for repeat-dose studies is 6 hours/day[8]
Facility costs	• Additional facility costs associated with providing the compressed air, chamber extract and inhalation equipment and protecting staff from the inherent safety risks of aerosolizing test articles with engineered solutions • Larger animal rooms for the same number of animals (2–3 times larger) • Additional cleaning costs for inhalation equipment as it is not feasible to discard this, plus analysis to ensure the next study is not compromised
Aerosol technology expertise	• Technical expertise to repeatedly aerosolize the test article close to target and ensure particle size is within required range
Analytical support	• A requirement for supplementary analytical support – this could mean daily support and at least 168 aerosol samples and four particle-size samples per week
Data requirements	• Additional raw data requirements, including the collection of exposure operation data

Regulatory authorities' requirements and exemptions for studies

5. The ECHA has requested that we should conduct a definitive OECD regulatory study for our chemical. Is that all that is needed?

It is common for regulatory bodies, for example the ECHA, to specify only the definitive OECD regulatory study in any guidelines, correspondence or feedback it supplies in response to submission for registration under REACH.

Nevertheless, it is usually necessary to perform a preliminary study with a dose range-finding study in advance of conducting the regulatory study, unless data exists that could be used to negate this requirement.

Furthermore, the ECHA is increasingly requesting additional endpoints that are non-standard, for which they give little or no detail. For this scenario, Labcorp can help you with the discussions with regulatory authorities that are advocated in advance of the study start as part of the enquiry process.

6. If the OECD study is submitted, will it be accepted by regulatory authorities in all countries?

In nearly all instances, yes, it will be accepted in all OECD member countries. However, there are exceptions depending on the country, the appropriate regulatory agency and the type of test article, so these will need to be checked before a study commences.

An example of this is that the JMAFF recommends 5 days/week for repeat-dose agrochemical studies.[9] However, the JMETI recommends 7 days/week for industrial chemicals. This is because these test articles come under the auspices of the JMLHW, which classifies them as quasi drugs.

7. When might a repeat (or additional) inhalation study be required?

Answering several key questions about the original study conduct establishes whether a repeat or additional study is required.

- Was the original study suitable?
- Will it meet regulatory requirements in relevant countries?
- What was the study design? Was it performed to GLP standards?
- When was it conducted?
- Was it conducted with different formulations?

Most OECD member countries accept OECD studies but, as discussed earlier, there are national differences depending on a country's regulatory agencies and the type of test article. Furthermore, any regulatory agency is likely to reject studies that have not been performed to GLP standards.

Guidelines are updated frequently to keep up with current thinking and safety concerns. For example, if the study was conducted ≥20 years ago, it was not uncommon to report and establish endpoints based on nominal concentration (i.e., the mass of generated test article divided by the total volume of air passed through the chamber system). The actual aerosol concentration may be anything from 2% to 60% of nominal aerosol concentration. Current guidelines for regulatory submission for renewals require the aerosol concentration (the concentration of the test article at the animal's breathing zone in an inhalation chamber) to be reported.[3]

For a different formulation, existing data must be reviewed to assess whether the formulation will produce a different outcome. This requires searching resources such as the ECHA database for previous studies.

8. Which guidelines have recently been updated and why?

For inhalation studies, the OECD 412 (4-week) and 413 (13-week) guidelines were significantly revised in 2018.[8,10] Unlike their 2009 iterations, the OECD 413 is no longer similar to the U.S. EPA OPPTS 870.3465 13-week guidelines.[11]

The driver for the update was the need to better evaluate the risk of nanomaterials, which may exhibit different toxicity based on their different physicochemical properties. Additionally, the update aimed to encourage manufacturers to have a better understanding of the mechanisms of toxicity of their material.

Among the many revisions, probably the most notable is the reduction in the acceptable MMAD. This is now ≤2 µm in contrast to the previous 1–3 µm. If this particle size cannot be achieved, preliminary characterization data is required in justification.

There is also emphasis on broadening methods for quantitative particle exposure (particle counts, size distribution or mass). This is challenging in regulatory compliance because it can require equipment that is cost prohibitive and is only available in academic institutions, which are usually not GLP compliant.

Other revisions include:

- Addition of the collection of BALF) to evaluate cellular and molecular components
- Greater emphasis on recovery of animals to evaluate lung burden measurements and clearance kinetics, which could mean a recovery period of up to 52 weeks with nanomaterials
- Encouragement for including pulmonary function and body temperatures to assess response to the test article
- Option to include additional parameters assessing toxicity, e.g., cholinesterase or bone marrow cytology

See Question 19 for more on particle size

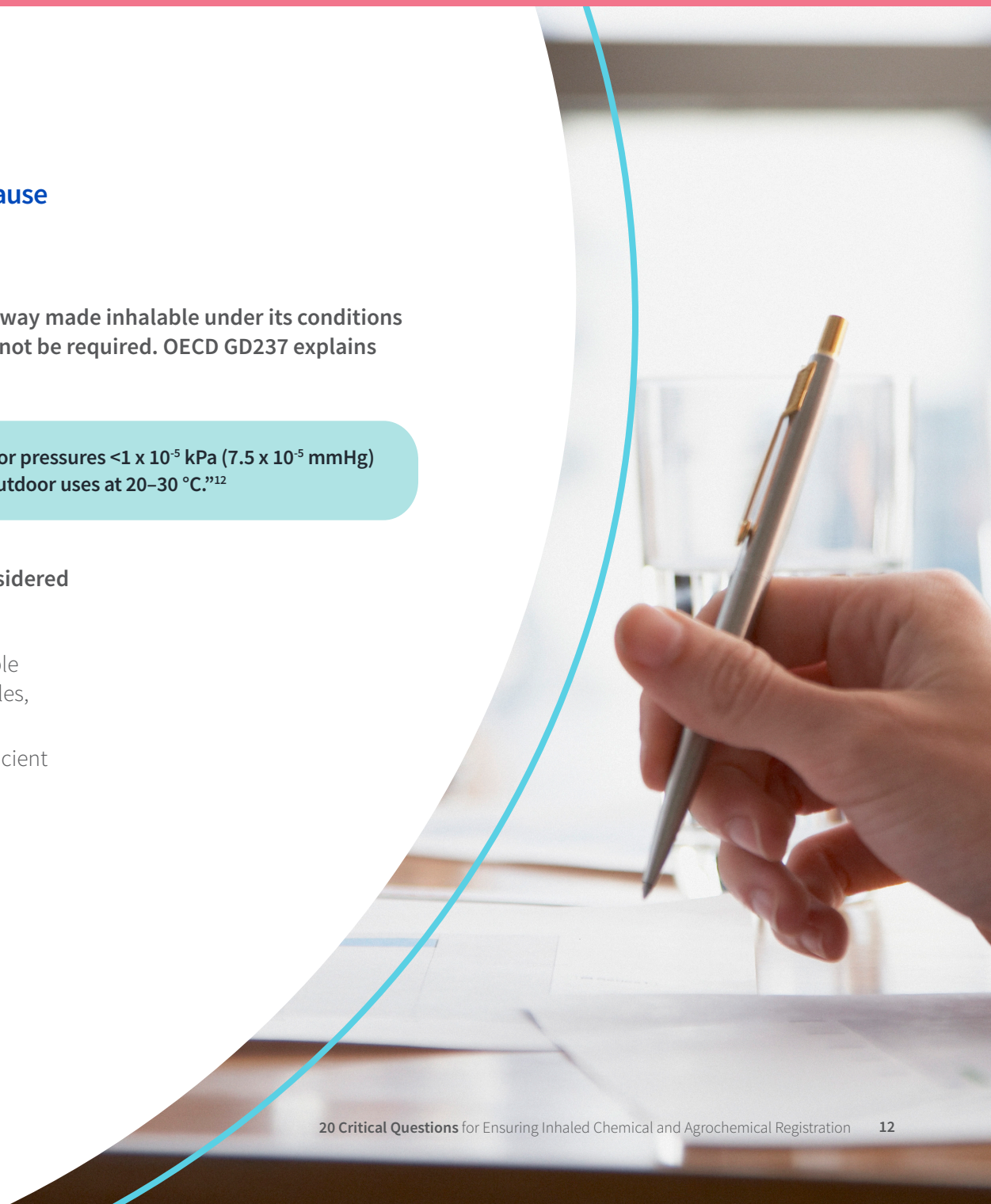
9. When is an inhalation study not required because of the test article's volatility or particle size?

If volatility is very low, or the test article is not in any way made inhalable under its conditions of use, storage or transport, an inhalation study may not be required. OECD GD237 explains the criteria for how a waiver can be requested:

> **"Low-volatility test chemicals are defined as having vapor pressures <1 x 10^{-5} kPa (7.5 x 10^{-5} mmHg) for indoor uses, and <1 x 10^{-4} kPa (7.5 x 10^{-4} mmHg) for outdoor uses at 20–30 °C."[12]**

Waivers for acute inhalation studies may also be considered for test articles that:

- Are too large to be inhaled (e.g., granules) and not liable to attrition and crumbling to produce inhalable particles, i.e., >99% is >100 µm in diameter
- Cannot be generated as a gas, vapor or aerosol in sufficient concentration to elicit animal toxicity in the optimal conditions of an inhalation chamber



Study design and data interpretation

10. Which laboratory species are usually dosed by inhalation?

To fulfill the OECD guidelines for chemicals and agrochemicals,[6] the preferred species is the rat, unless specified to the contrary by the regulators. Other species may include mice or rabbits (e.g., for inhalation reprotoxicology studies such as OECD 414: Prenatal Developmental Toxicity Study[13]).

See Questions 4 and 12 for more on differences between studies via the inhalation route and oral and dermal routes

11. What are the main differences in the study design between oral and inhalation studies?

Oral dosing tends to be bolus dosing and the animals are dosed individually, as they are for the dermal route. However, for inhalation exposure, the animals are dosed as a group and exposure is usually performed over several hours, as shown in Table 2.

Table 2: Inhalation exposure.

Type of exposure	Acute	Subacute and Subchronic
Test number	OECD 4034 and 4331	OECD 4128 and 41310
Study duration	1 day	28 and 90 days
Exposure to aerosol	Usually 4 hours	Up to 6 hours

Extensive respiratory tract pathology is also required to elucidate possible local effects of inhaled test articles.

There is also a greater need for analytical support for inhalation studies: both aerosol concentration and particle-size samples should be analyzed daily throughout the duration of an inhalation study.[3] It is not necessary to analyze samples with this frequency in oral studies. Dose precision and calculation are far more complex with inhalation because the animal is in effect self-dosing. Its own tidal volume and breathing rate influence the dose received from the atmosphere containing the test concentration.

Engineering and procedural controls are particularly necessary due to the inherent risks of aerosolized test articles. Specialized safety cabinets are required, and additional precautions are needed for nanomaterials and for powders with explosive potential.

12. What other differences are there between oral and inhalation studies?

The four key areas of difference, relating to the need to deliver a steady dose of test article in a stable atmosphere over a protracted time period, are described in Table 3.

Table 3: Differences between oral and inhalation studies in relation to delivery of a test article in inspired air.

High quantity of test article	• The required quantity is greater for an inhalation study than for oral studies. Maximum target doses may be limited by the aerosol concentration that it is practical to generate, or that can be generated while at the same time achieving a regulatory-acceptable particle-size distribution • See Question 19 for more on particle size
Dynamic nature of exposure	• The exposure system for inhalation safety testing is dynamic, constantly generating an aerosol during a protracted exposure duration. This requires significantly more of the test article than is needed for other routes of administration, even though there are multiple ways of reducing the amount of test article that is necessary ('test article conservation')
Aerosol generation efficiency	• Typically, the efficiency of generation for most aerosols is 60% or less. Accordingly, the default test article requirement for a standard study under OECD 403 or 433 guidelines is up to 1 kg or 1 L of formulation;[1,4] it is not uncommon to require several kilograms for repeat-dose studies. In this respect, it is noteworthy that the amount of test article required for a snout-only study is significantly lower than required for a whole-body study • See Question 14 for more on whole-body vs. snout-only studies
Atmosphere control	• The guidelines[3,11,14] state: • Adequate air exchange rates of at least 2–3 times the respiratory minute volume of animals exposed for snout-only studies • A dynamic airflow of at least 10 air changes per hour for whole-body studies • In the case of whole-body inhalation studies, the total volume taken up by the test animals should not exceed 5% of the chamber volume; this contributes to stability of a chamber atmosphere

13. Is a control group necessary for an acute inhalation study?

A concurrent negative (air) control or vehicle (water) group of study animals is not usually necessary for acute inhalation studies. However, when a vehicle other than water is required for generating the test atmosphere, a vehicle control group may be added depending on which vehicle is chosen.

14. Should the study be conducted using a whole-body or snout-only chamber?

Both the OECD[3] and U.S. EPA guidelines[11,14] indicate that snout-only exposure should be used as default unless there is specific justification to the contrary. The rationale for this is given in Table 4.[3]

Nevertheless, there are disadvantages to the snout-only method in some circumstances, as listed in Table 5, and these must be taken into consideration. The guidelines state the decision depends on whether specific objectives of the study may be better achieved by using whole-body exposure depending on the scenario, in which case this exposure method should be justified in the report.

Table 4: Reasons why snout-only exposure is preferred over whole-body exposure.[3]

Exposure/uptake by other routes	Minimized – exposure via oral route via preening or dermal route are relevant concerns when testing aerosols
Quantity of test article	Lower than whole-body exposure because test chamber volume and the airflows required are considerably lower
Containment of test article	Easier than in whole-body studies
Achieving test concentration	High concentrations are readily achieved (e.g., limit concentrations)
Test article stability and test atmosphere in-homogeneity	Minimized due to lower chamber volume
Time required to attain inhalation chamber equilibration (t95)	Negligible relative to exposure duration
Option for multiple exposure durations in a single test	Adding or removing animal restraining tubes during exposure to a fixed steady-state chamber concentration allows for multiple exposure durations in a single test (the OECD 403 alternative "C [concentration] x t [time] protocol", utilizing the same exposure concentrations for multiple exposure durations[4])
Animal welfare	Exposure of individual animals can be interrupted at any time during the course of exposure to ensure animal welfare integrity
Ease of physiological observations	Animals are readily accessible for specific physiological measurements (e.g., respiratory function, body temperature) or the collection of blood, if applicable

Table 5: Disadvantages to snout-only exposure.

Animal welfare	• No food or water during exposure • Suffocation risk if animals try to turn in tubes (more likely with younger animals or during acclimatization) • Stress, particularly with the required 6-hour exposure time
Test practicalities	• Restricted view of animals, making behavioral observations difficult • Achieving low exposure levels can be technically challenging • More labor intensive than whole-body studies

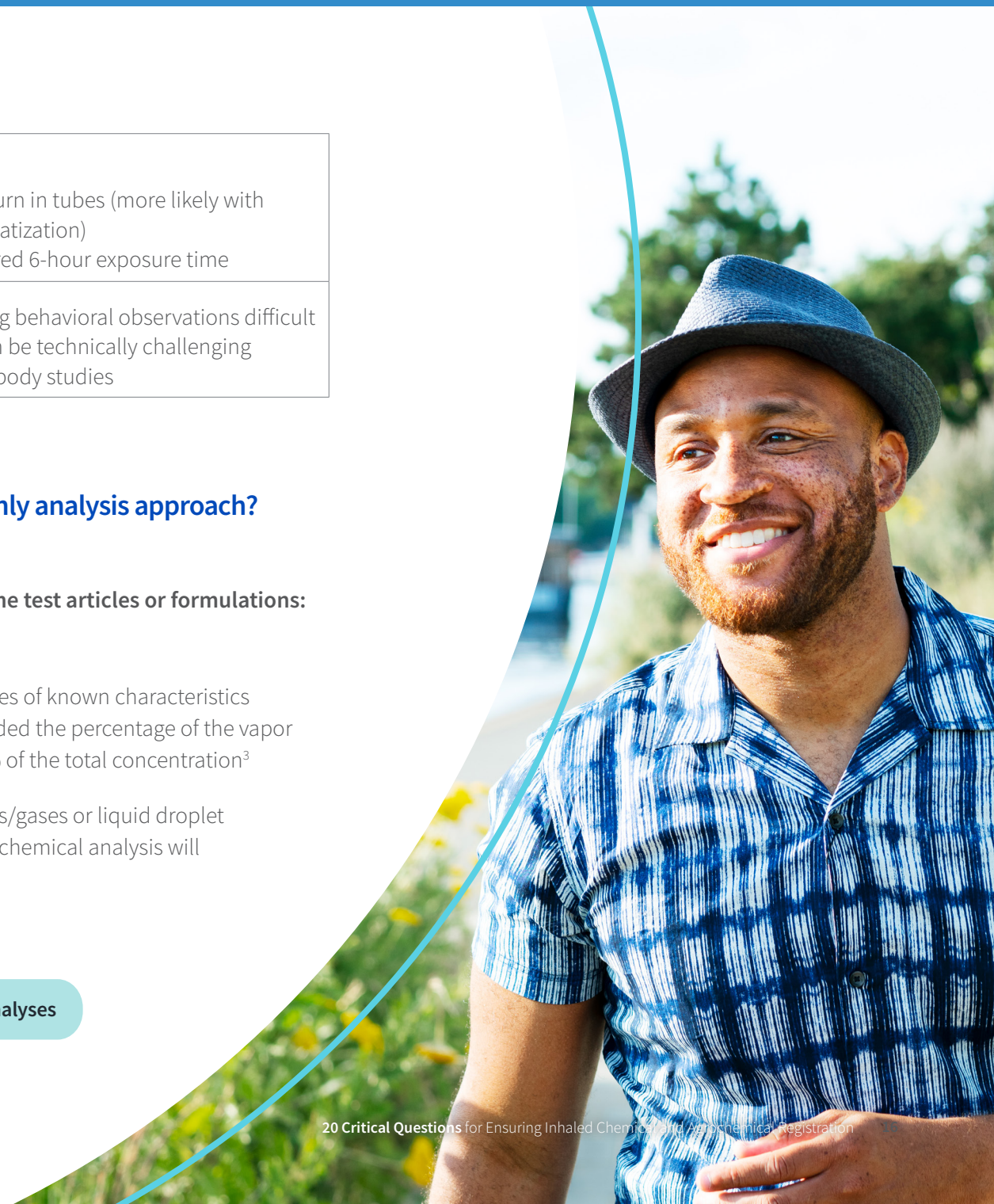
15. What situations can employ a gravimetric-only analysis approach?

Gravimetric-only analysis is acceptable only for some test articles or formulations:

1. Single-component powder aerosols
2. Aerosols of low-volatility liquids (e.g., simple mixtures of known characteristics such as pesticide and chemical formulations) provided the percentage of the vapor phase under testing conditions does not exceed 1% of the total concentration[3]

For very complex or non-homogenous mixtures, vapors/gases or liquid droplet aerosols, gravimetric analysis may not be feasible and chemical analysis will be required.

See Question 18 for more on gravimetric and chemical analyses



16. Does the first inhalation study with analytical support need to be GLP compliant?

Yes, if the requirement is for only one study to be conducted for registration, e.g., an acute study. Whether validation of method has to be GLP compliant depends on local quality assurance (QA) because good practice may be covered by QA during routine process inspections. Validation of method for aerosol concentration and particle-size analysis is summarized in Table 6.

Table 6: Aspects of method validation for aerosol concentration and particle-size analysis (depending on local QA).

- Specificity of the test article
- Limit of detection
- Linearity of the standard curve
- System precision (repeatability)
- Test article recovery from the collection media
- Sample stability

If the requirement is for a repeat-dose study, (i.e., a subacute or chronic study), then the answer is “no” as with any other route of administration, because good practice is covered by QA during the routine inspections.

Early dose range-finding/preliminary studies do not have to be GLP compliant unless formulation stability data is required for dose formulations. Inhalation studies are rare for formulations. Usually a formulation is used as supplied unless target aerosol concentrations are technically challenging to achieve and dilution is required to make aerosol delivery more manageable. For these rare situations, formulation stability and homogeneity would have to be assessed.

Labcorp studies are conducted to a single standard and therefore the only difference between non-GLP and GLP studies is the absence of direct QA oversight.

17. What work is performed during the preliminary exposure system characterization trials and why is it necessary?

Technical pretest or characterization of an exposure system is a requirement specified in the OECD GD39 guidelines.[3] This work is conducted in advance of animal dosing and has two goals.

Primary goal: to confirm the suitability of the test article generation system, whether an atomizer/nebulizer for liquids, a powder disperser for solids or a dilution system for gases.

The operating parameters of the selected generation system are based on the number and species of animal and the exposure system; they usually differ between studies.

Once they have been established:

Secondary goal: to demonstrate, by analysis of the atmospheres produced, that there is satisfactory control over the system in terms of stability and reproducibility of concentration, particle-size distribution and spatial homogeneity within the test chamber.

For quantification of atmosphere concentrations, the method of analysis for each test article is validated as part of this investigation. Related to this, the operating characteristics of the exposure system may need to be adjusted when animals are introduced into the system because they have a filtering effect as well as introducing moisture into the chamber.

These changes can be predicted with reasonable precision, but it may be necessary to make minor adjustments during the first few days of treatment.

See Questions 18 and 19 for more on concentration and particle size

18. How is aerosol concentration determined?

Test guidelines require samples to be collected directly from the exposure system from a location that is representative of the breathing zone for the animals (generally, the restraint tube attachment position). The sample collection system is selected to provide optimal trapping for the test article and to permit any scheduled analysis of the active component.

If the test article produces a mist or powder aerosol, the concentration can be calculated gravimetrically, based on the weight gain of the sample media, or chemically, by analysis using the methods developed in the characterization trials or by both methods.

Gravimetric analysis is typically conducted either during or immediately after the completion of each exposure period; this provides rapid feedback to staff regarding the delivered aerosol concentration.

Chemical analysis, if performed, can be conducted daily in the early stages of a study until it is clear that: (1) delivered concentrations are consistent with protocol-defined targets; and (2) adequate control over the exposure conditions is being maintained. The frequency is then commonly reduced or analyses are batched together (typically two or three occasions per week depending on the study duration).

If gravimetric analysis is not possible (e.g., vapors/gases), daily chemical analysis is required throughout the study duration.

The choice of sampling technology depends on the characteristics of the test article. Usually, glass fiber filters or sintered traps are the methodologies of choice for most liquid or powder aerosols. However, for powder aerosols with very low concentrations, PTFE filters may be required, as is the case for nanomaterials. Particle size sample collection is principally carried out using multistage cascade impactors, but it can be undertaken with an APS based on an optical (laser) system. However, extrapolating the value presented using the APS to the MMAD is challenging.

See Question 15 for criteria for gravimetric and chemical analyses

19. What should be the target MMAD?

For testing aerosols, a respirable particle size must be achieved. The required MMAD is clearly defined in the OECD GD39: Guidance on Inhalation Toxicity Studies as ≤4 µm for acute/single-dose and ≤2 µm for repeat-dose studies to maximize pulmonary deposition,[3] with a recommended GSD in the range of 1–3.

Even when the particle size of the input material is greater than this required range, once generated into a stream of air, the larger particles tend not to be aerostable and will sediment from the airstream before they reach the breathing zone of the animals. Exposure systems are designed to encourage this so that only particles of appropriate MMAD reach the animal's breathing zone.

If the MMAD of the particulate aerosol still exceeds the target range after adjusting for this effect, then the particulate material can be subjected to mechanical processes to achieve the required particle-size distribution, e.g., by milling. However, care has to be taken not to decompose or alter the test article. If mechanical processes or the aerosolization process still results in a MMAD above the target range, then the target concentration may have to be lowered.

Achieving the target MMAD may be unfeasible and unrealistic. When this is the case, the study report must include additional justification that attempts were made to reduce the MMAD and the study otherwise complies with the guideline. Discussions with the regulatory agencies may be needed when this occurs.[3]

20. Why do the delivered inhalation aerosol concentrations vary from the target values?

According to OECD GD39[3], ideally, chamber concentration should be maintained so that the aerosol samples taken from the breathing zone do not deviate from the mean chamber concentration by:

- More than ±10% for gases and vapors
- More than ±20% for liquid or solid aerosols

Nevertheless, the regulators also recognize that this cannot always be achieved.

Unlike dispensed volume methods used for other administration routes, inhalation exposure involves a number of variables. Seemingly minor changes in the exposure system, such as exposure environment, including air temperature, humidity and atmospheric pressure, can impact the achieved aerosol concentration in the breathing zone of the animals. These variations occur even in the absence of alterations to system settings. Additionally, there is always a proportion of measurement variation due to the relative precision of sample collection and analysis methods.

In addition to these uncertainties, the animals themselves have a direct effect on concentration because they are filtering the atmospheres they are exposed to and are supplementing the moisture level. There are also highly variable breathing patterns between individuals.

A fundamental aim in inhalation studies, therefore, is to monitor and control as many of these variables as possible in order to minimize the differences between doses delivered to individual animals.

Summary

In summary, there are many aspects that need to be considered in achieving a successful and regulatory-approved study. Before you start your study, you need to:

- Assess the regulatory requirements and apply them to your test article
- Consider the physicochemical properties of your test article
- Plan studies in detail within your wider testing program and allow sufficient time for conducting studies and reporting
- Validate the gravimetric and/or chemical analytical methods for monitoring
- Perform preliminary characterization trials to determine the concentration and particle size produced by the aerosol-generation system
- Adjust the exposure system to compensate for the impact of introducing an animal to it
- Schedule regular observations and sampling, with animal welfare being paramount
- Initiate postlife investigations and interpret the results

Labcorp has the capabilities and experience to ensure all this can happen.

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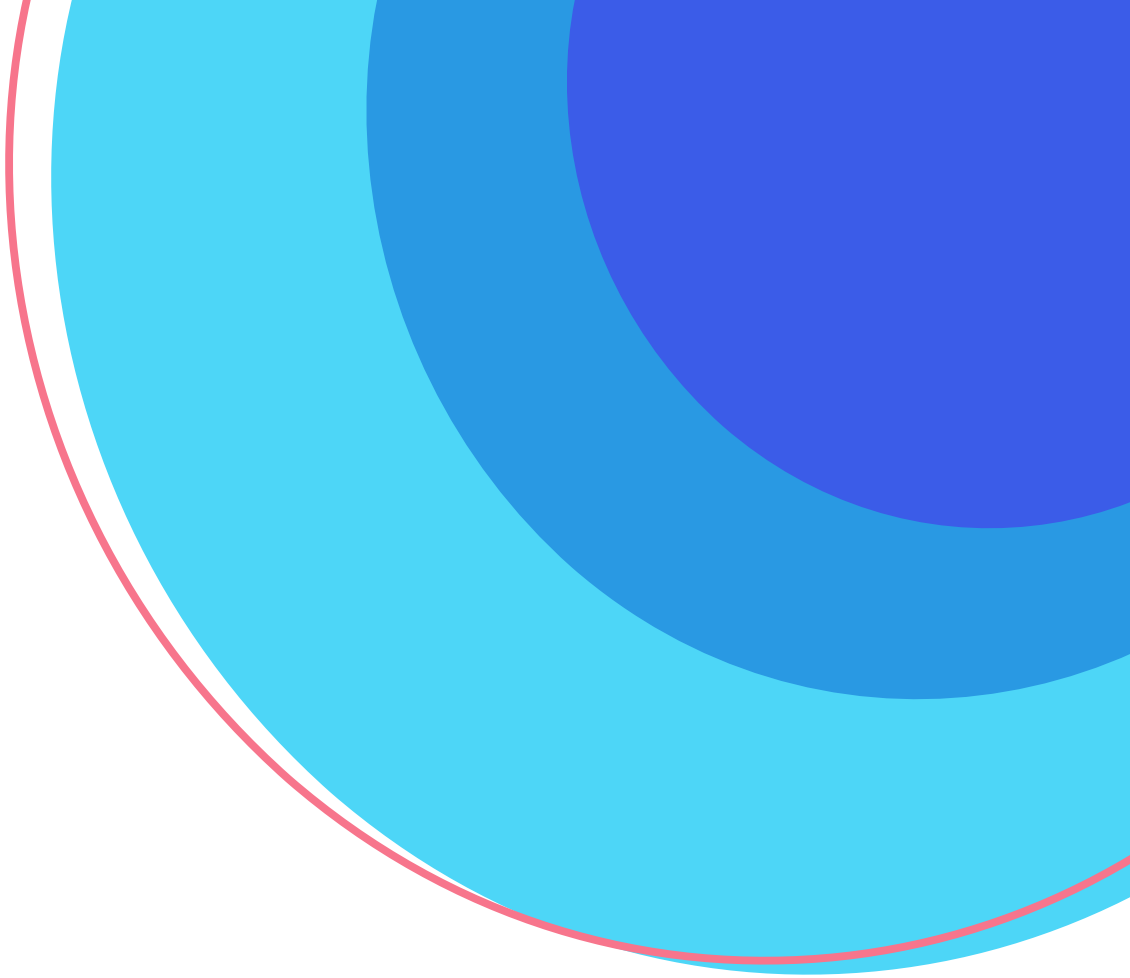

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