EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection
Unit: Toxicology and Chemical Substances
European Chemicals Bureau
I-21020 Ispra (VA) Italy

Methods for the Determination of the Hazardous Properties for Human Health of Man Made Mineral Fibres (MMMF)

Edited by:

David M. Bernstein
Juan M. Riego Sintes

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I. Introduction/Background

In December 1998 the Commission adopted Directive 97/69/EC adapting to technical progress for the 23rd time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. Directive 97/69/EC establishes in its nota Q criteria to exonerate synthetic mineral fibres (MMMF) from classification as carcinogens on the basis of the results of four tests.

However, Annex V to Directive 67/548/EEC does not yet include suitable testing methods to determine the hazardous properties of MMMF.

From 1996 to 1998, extensive discussions took place in several meetings chaired by the European Chemicals Bureau (ECB) on different possible schemes for classification of MMMF and the instruments needed to effectively implement them. The discussions involved several expert groups, the National Co-ordinators for Testing Methods of Annex V to Dir 67/548/EC and the Classification and Labelling Working Group on CMR substances. As an outcome of these discussions, four protocols to determine the possible hazardous properties of MMMF for human health were produced. These protocols were developed in order to fulfil the requirements of nota Q of Dir 97/69/EC.

Although there was a general agreement among the experts that these protocols are not yet ready to be introduced into Annex V of the Directive, there was also a general consensus that they represent the way forward in testing of synthetic mineral fibres and their use is recommended for testing of such fibres.

A compilation of these protocols is presented here in order to facilitate their consultation and use by interested parties. Comments and information on experiences when actually using them should be addressed to the ECB (see address in page 93) and would be gratefully appreciated.

The protocols are described in documents

- ECB/TM/26 rev. 7: Biopersistence of Fibres. Short Term Exposure by Inhalation
- ECB/TM/27 rev.7: Biopersistence of Fibres. Intratracheal Instillation
- ECB/TM/18(97) rev. 1: Carcinogenicity of Synthetic Mineral Fibres after Intraperitoneal Injection in Rats
- ECB/TM/17(97) rev. 2: Chronic Inhalation Toxicity of Synthetic Mineral Fibres in Rats

that are reproduced in chapter 2.

Additionally, the EU Member States representatives recommended the development of a testing protocol for a sub-chronic (90 days) inhalation toxicity test that could eventually replace the chronic test, accordingly a protocol for this test was also produced:

- ECB/TM/16(97) rev. 1: Sub-chronic Inhalation Toxicity of Synthetic Mineral Fibres in Rats

As additional information we have also included in Annex 1 some recommendations made by an ad-hoc experts' group as a Guidance note on Counting and Sizing of fibres (Document ECB/TM/21(97)). A list of meetings in which the protocols were presented and/or discussed is shown in Annex 2. The participants to these meetings are listed in Annex 3.

II. Protocols

II.1 Biopersistence of Fibres. Short Term Exposure by Inhalation (ECB/TM/26 rev. 7)

B.XX. Biopersistence of Fibres. Short Term Exposure by Inhalation

1. METHOD

1.1 INTRODUCTION

See General Introduction Part B*(in Annex V to Dir 67/548/EC)*.

1.2 DEFINITIONS

1. See General Introduction Part B in addition to the following.
2. *Biopersistence*: The ability of a material (fibre) to persist in the lung in spite of the lung's physiological clearance mechanisms and environmental conditions.
3. *Fibre* (also referred to as fibrous particles): An object with a length to width ratio (aspect ratio) of at least 3:1.
4. *WHO fibre*: A fibre with a length greater than 5 μm and a diameter less than 3 μm (reference (1)).
5. *Particle* (also referred to as non-fibrous particles): An object with a length to width ratio of less than 3:1.

1.3 PRINCIPLE OF THE TEST METHOD

The objective of this method is to assess the in-vivo pulmonary biopersistence of inhaled fibrous and non-fibrous particles in the rat following repeated exposure for 5 days. This method is designed for the evaluation of synthetic mineral fibres, however, it can be applied with appropriate modification to organic and natural fibres.

This testing protocol is intended to be used as part of a tiered approach to the evaluation of fibres. This protocol evaluates the pulmonary biopersistence of fibres as a function of fibre length, however, it does not evaluate the effect of fibre diameter on human pulmonary deposition.

The specific use of the clearance of fibres longer than 20 μm in length in this protocol is designed to reflect the removal of fibres by dissolution and disintegration and is considered to be analogous in both humans and rats.

1.4 DESCRIPTION OF THE TEST METHOD

Laboratory rats are exposed by inhalation for 6 hours/day for 5 consecutive days to well characterised fibre test atmospheres which have been optimised to be largely rat respirable. Following the end of the exposure period, subgroups of animals are sacrificed at pre-determined intervals and the lung burden determined by suitably validated extraction and measurement methods.

Measurements include characterisation of the number, bivariate size distribution and chemical composition of fibres (and particles) in the lung at predefined intervals following the cessation of the last exposure and determination of the time for removing 50% of the fibres ($T_{1/2}$) which are longer than 20 μm. The $T_{1/2}$ of other length fractions: <5 μm, 5-20 μm, WHO fibres should also be determined from the data.

The procedures in this test method have been described in references (2), (3), (4) and (7).

1.4.1 Preparations

Healthy young adult animals are assigned to the control and treatment groups by randomisation by weight. The animals are kept in their cages for at least 5 days prior to the start of the study to allow for acclimatisation to the laboratory conditions. During this period, the animals should be placed in the nose-only restraint tubes on the exposure system for 6 hours/day for at least 4 days and exposed to filtered air at similar conditions that will be used during the study.

The fibre under test is administered by inhalation of an atmosphere of aerosolised fibres. The method of aerosolization is dependent on the physical chemical properties of the fibre. The method chosen should not contaminate the fibres and it should minimise possible alteration of the fibre surface or the production of non-fibrous dust (through excessive grinding or abrasion of the fibres).

1.4.2 Test conditions

1.4.2.1 Experimental animals

Rat, Fischer 344 or Wistar, supplied specific pathogen free (SPF), virus antigen-free (VAF+) and maintained under optimum hygienic conditions (OHC). Upon receipt and at selected intervals throughout the study, a sentinel group of rats should be analysed for bacteriological and viral contamination. Another strain of laboratory rat may be used providing it is validated in comparison to the Fisher 344 or Wistar.

The animals should be maintained under barrier conditions throughout the study. The rats should be acclimated to the animal room used for housing during the test for at least 5 days after clinical health examination. The rats should be approximately 8-10 weeks old with a weight range of ± 20 % of the mean at the start of the acclimation period. The exact age and weight should be recorded as part of the study data.

1.4.2.2 *Number and sex*

At least 7 rats (either male or female) /group/sacrifice time point should be used. One sex only is used as no difference has been reported in the response to chronic fibre inhalation in male and female rats. In order to eliminate deviant values the lung fibre content should be evaluated statistically to remove up to two extreme outliers if necessary. If additional end points are planned, the number should be increased by the number of animals scheduled to be killed before the completion of the study. For the control group, five animals / group / sacrifice time point should be used.

1.4.2.3 *Animal room and housing during non-exposure periods*

Animals should be housed in stainless steel wire or polycarbonate cages. During the exposure period, cages for housing when the animals are not being exposed should be arranged in such a way that possible effects due to cross contamination of fibres from one group to another are minimised. If 2 or more test fibres are included in the study, each fibre group should be sufficiently isolated to minimise cross contamination between groups.

1.4.2.4 *Choice of Exposure Concentrations (Dose Levels)*

Animals should be exposed to a single concentration of the test substance. The fibre exposure aerosol should be prepared to be rat respirable and have:

- A mean aspect ratio of at least 3:1,
-
- At least 100 fibres/cm^3 longer than 20 µm in length, if technically feasible,

It should also be ensured that:

- The exposure concentrations stated below refer to the number of fibres with geometric mean length greater than 20 µm. The geometric mean diameter of those fibres longer than 20 µm should be as close to 0.8 µm as possible, for fibres with a density $\rho \cong 2.4$, if technically feasible. For fibres with densities different from this the corresponding GMD should be determined. (Note: The GMD varies as the square root of the density for a constant median aerodynamic diameter).
- The gravimetric mean concentration of those fibres, which are 0.8 µm or less, should not exceed 40 mg/m^3 in the exposure aerosol, if technically feasible.
- As an upper limit, the gravimetric concentration of all particles (fibrous and non-fibrous) in the test atmosphere should not exceed 60 mg/m^3, if technically feasible.

The typical range of exposure concentrations resulting from the above conditions correspond to an approximate mass burden of 0.5 to 1.0 mg following five days of exposure to insoluble fibres.

A control group of animals exposed to filtered air only is to be included and analysed. The treatment of the control animals should be performed under the same conditions as for the animals receiving the test fibres.

It should be noted that diameters could be smaller than 0.8 μm if justified by the dimensions of the bulk fibre.

1.4.2.5 Characterisation of the Test Article used for aerosol generation

The chemical composition of the fibre material supplied for testing at least to within 0.5 % and the density of the fibres should be provided.

1.4.2.6 Duration and Frequency of Exposure

Each group of animals should be exposed for 6 hours/day for 5 consecutive days.

1.4.2.7 Observation period

Sub-groups of animals should be sacrificed at a sufficient number of intervals after cessation of exposure so as to permit good definition of the clearance curves and calculation of the clearance half-times for each length fraction. The following sacrifice intervals have been used frequently in published biopersistence studies and are provided so as to permit cross comparison between studies:

Sacrifice intervals: 1 day, 2 days, 3 days, 14 days, 4 weeks, 3 months, 6 months, 12 months.

All studies should include at least sacrifice intervals at 1 day, 2 or 3 days, 14 days, 4 weeks and 3 months. Additional sacrifice intervals can be included as considered necessary to obtain further definition of the shape of the clearance curve. The background level/limit of detection for the fibres in the treated lungs should be determined based upon the control lungs. At each sacrifice, if an exposure group has a mean concentration of fibres >20 μm in length which is less than 5 % of the number found on day 1, the remaining animals in that group should be terminated without further analysis.

1.4.2.8 Laboratory Validation Fibre

Prior to starting studies on test fibres, laboratories with no previous experience in testing fibres should analyse a validation fibre using a similar exposure regime. All laboratories should analyse the validation fibre according to this protocol if no similar test has been carried out in the last 5 years. Fibres such as E-glass, MMVF21 or MMVF 10a are recommended as the validation material. Optionally other fibres could be used as validation material if sufficient data are available.

1.4.3 Procedure

1.4.3.1 Test Article Preparation

In order to achieve the requirements stated in section 1.4.2.4, the bulk fibre used for aerosol generation should be prepared or pre-selected by size to be respirable in the rodent. As a general guideline, for fibres of density $\rho \cong 2.4$, a geometric mean diameter as close to 0.8 μm as possible and a geometric mean length of approximately 15 μm will facilitate achieving those requirements.

If a pre-selection process is used or the bulk fibres used are produced by a non-commercial production method, a validation must be included in the study report which shows that the fibre has similar chemical and surface characteristics as compared to that produced commercially.

1.4.3.2 Fibre Aerosol Generation system

The fibre aerosol generation system must be capable of producing the required aerosol concentration of fibres as described above continuously for a period of 6 hours/day without contaminating the fibres, altering the fibre surface or producing non-fibrous dust (through grinding or abrasion of the fibres). A suitable charge neutraliser (e.g. Ni63) should be placed immediately following the aerosol generator to assure that the fibres are discharged to Boltzmann equilibrium.

The system should be able to produce the required aerosol exposure with a mean uniformity of plus or minus 15 % based upon gravimetric measurements of aerosol concentration.

1.4.3.3 Inhalation Exposure System

It is recommended that the flow-past nose-only exposure system (reference (3)) be used. Other nose-only exposure systems may be used if they are validated with respect to equivalent or greater fibre deposition of fibres > 20 μm in length as compared with the flow-past system.

The testing facility must provide documentation showing that the uniformity of fibre concentrations at the top, middle and bottom level of the exposure system is within ± 15 %.

1.4.3.4 General observations

1.4.3.4.1 MORTALITY

All animals should be observed for mortality/moribundity before the start and after the completion of each exposure and at least once on non-administration days.

1.4.3.4.2 CLINICAL SIGNS

Each animal should have a detailed clinical observation for signs of toxicity, including time of onset, intensity and duration:

- once during acclimatisation phase
- twice daily (once prior to the daily exposure) during the 5 treatment days
- once daily during the first two weeks after end of the exposure phase
- once weekly thereafter.

1.4.3.4.3 BODY WEIGHT

All animals should be weighed at least

- once at the beginning of acclimatisation period,
- on the day of first exposure, prior to the start of exposure,
- once weekly thereafter through week 12,
- once monthly thereafter.

1.4.3.4.4 GROSS NECROPSY

As a control of animal health, all animals in the study should be subjected to a full, detailed gross necropsy, which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. All animals should be anaesthetised and sacrificed by exsanguination. All gross necropsy findings in the lungs should be recorded.

1.4.3.4.5 ORGAN SAMPLING

Animals found dead or sacrificed moribund should be autopsied but their lungs not sampled for fibre analysis.

In a minimum of 5 animals/group per sacrifice:

- The lungs and the lower half of the trachea will be sampled.

Using a dissecting microscope:

- The lower half of the trachea and the main stem bronchi should be resected in one piece from the lung lobes, cleaned from remaining mediastinal tissues, weighed and immediately deep-frozen.

- The lung lobes should be weighed together and immediately deep-frozen.

The lungs should be frozen on dry ice and then stored at -20°C. All lungs should be freeze dried or critical point dried as quickly as possible thereafter and within two weeks of sacrifice to minimise possible fibre dissolution. Other appropriate methods can also be used if properly validated.

1.4.3.4.6 FURTHER PROCESSING OF LUNGS

The 7 animals allocated for sacrifice in each fibre group at each time-point are allocated for lung digestion and subsequent fibre analysis.

Following drying of the rat pulmonary lobes, the dry lung weight should be determined. The dry tissue should then be digested using an appropriate method. For mineral fibres the preferred method is low temperature plasma ashing.

All tissue digestion methods should be validated prior to use. Using a standard addition procedure and a minimum of 3 previously unexposed rat lungs / dose group, doses of 0.05, 0.1 and 0.5 mg of a similar well characterised fibre should be injected and the lungs digested. The mean fibre number should be within ± 25 % of what was injected and the size distribution recovered should not be statistically different from that injected.

1.4.4 Study Monitoring

1.4.4.1 Exposure system monitoring

- Airflow rate (monitored continuously and recorded at least once per day).
- Oxygen concentration. The oxygen concentration in the vicinity of animal's nose should be maintained at a level of at least 19.5 %. If the flow-past nose-only exposure system is used and the airflow supplied to each animal is at least 1 l/min, then it is not necessary to measure the oxygen concentration.
- Temperature & humidity of the air supply (at least once per day). The temperature should be maintained at 22 ± 2 °C. To achieve the fibre aerosol exposures, it is recognised that the supply air to the generator can be dry and as such no lower limit is placed upon humidity. Review of the air control groups from a series of chronic studies has shown that there is no adverse effect from such low humidity.

1.4.4.2 Exposure atmosphere monitoring and analysis

All sampling for measurement of the aerosol exposure concentration and size distribution should be performed near where the animal's nose would be in the exposure system.

For the air control group, the sampling duration should be as long as possible (approximately 3 to 5 hours) in order to permit the assessment of the absence of contamination.

Sufficient monitoring of the exposure atmosphere should be performed during the pre-study phase in order to assure that the required fibre aerosol concentrations and uniformity are achieved during the study. If a flow past exposure system is not used, sampling should be performed using methodology designed to minimise anisokinetic sampling errors.

The analyses specified below should be considered as the minimum analyses that should be performed. If anomalies in the results occur, additional filters should be analysed if available.

1.4.4.2.1 GRAVIMETRIC (mg/m^3)

If gravimetric concentration is used for monitoring of the aerosol fibre number, aerosol mass monitoring should be performed daily for a duration representative of the daily concentration. Daily sampling should be performed for at least 2 hours per day with each individual sample of at least one-hour duration. The gravimetric concentration should be determined from each filter sampled and expressed in mg/m^3.

1.4.4.2.2 1.4.4.2.2 FIBRE AND PARTICULATE NUMBER (fibres/cm^3) AND BIVARIATE SIZE DISTRIBUTION (µm) BY SCANNING ELECTRON MICROSCOPY (SEM)

These should be sampled at least twice per day. These samples should be taken in timely coincidence with the gravimetric sampling with sample duration dependent upon fibre type (usually less than 30 minutes). One filter per day should be analysed for fibre and particulate number, with the remaining filters used if anomalies are found. Bivariate analysis of diameter and length should be determined at least twice weekly with the additional filters used if anomalies are found. Fibre concentration should be expressed as total number of fibres/cm^3 and the number of fibres/cm^3 with length > 20 µm, 5-20 µm, < 5 µm and WHO fibres and the number of particles/cm^3.

1.4.4.2.3 CHEMICAL ANALYSIS

One filter sample should be taken for possible analysis.

1.4.4.3 Counting and Sizing Rules (for aerosol and lung fibres)

The general guidelines provided by the WHO/EURO (reference (1)) are recommended with the following additional procedures for mineral fibres (due to the possibility of smaller diameters, additional procedures may be required for natural or organic fibres).

1.4.4.3.1 LENGTH AND DIAMETER

Sizing of length and diameters should be performed using a SEM at a magnification of at least 2000. All objects which are seen at this magnification are to be counted. Fibres crossing the boundary of the field of view should be counted as follows. Fibres with only one end in the field are weighted as half of a fibre and fibres with neither of their ends in the field are not measured. Diameters of fibres which are seen at 2000 magnification should be measured at 'full screen' magnification (usually up to a magnification of 10,000). No lower or upper limit is to be imposed on either length or diameter. For bulk fibres with mean diameters below a few tenths of a micrometer, an initial magnification of at least 5000 should be used. The length and diameter are to be recorded individually for each fibre measured so that the bivariate distribution can be determined. When sizing, an object is to be accepted as a fibre if the ratio of length to diameter was at least 3:1. All other objects are considered particles. There should be no truncation in the measurements. If fibre measurements are made using SEM photomicrographs or video prints where the magnification of the photo or print is at least twice that of the SEM screen, then an initial SEM magnification of at least 1000 is acceptable, providing fibres of 0.1 µm diameter can be resolved. When using photomicrographs or video prints, higher magnifications should be used for diameter measurement than for length measurement in order to ensure good precision.

1.4.4.3.2 STOPPING RULES

Enough fields of view are to be counted for evaluation so that at least a total of 0.15 mm^2 of the filter surface (for 25 mm diameter) is examined. Once this condition is fulfilled:

1. Fibres: The evaluation of fibres should be stopped when 400 WHO (L > 5 μm, D < 3 μm) fibres are counted/measured for each sample (lung, filter, etc) analysed by SEM, or a total of 1000 fibres and non-fibrous particles were recorded, or 1 mm^2 of the filter surface was examined, even if a total of 400 countable WHO fibres was not reached. Otherwise, the procedural variability and counting errors could result in a false estimate of the measure. The total number of fibres per filter should be determined by normalising the surface area counted to the total surface area of the filter.

2. Particles: The recording of particles can be stopped when a total of 100 particles are counted. If the size distribution of particles is measured, care should be taken in the lung samples to confirm by EDAX which particles are of the same composition as the fibres.

Other strategies for measurement (such as size selective analysis using a minimum of 100 fibres per category with at least 3 length categories) can be used providing that the method has been validated to produce similar results statistically in comparison to the above method.

2. DATA

2.1 ANIMAL DATA

Individual data should be provided. Additionally all data should be summarised in tabular form showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons and the time of any death or humane kill, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the number of animals showing lesions, the type of lesions and the percentage of animals displaying each type of lesion. In addition, the body weights and lung weights should be provided.

2.2 FIBRE CHEMISTRY

The chemical composition of the fibre material provided for testing to within at least 0.5 % and the density of the fibre tested should be presented.

2.3 EXPOSURE AEROSOL

The following parameters should be reported for the exposure aerosol:

2.3.1 Fibres

Number of fibres evaluated microscopically; Mean and standard deviation gravimetric concentration (mg/m^3); Mean Total Fibres/cm^3; Mean WHO Fibres/cm^3; Mean number of fibres > 20 μm in length/cm^3; Mean number of fibres < 5 μm, 5-20 μm and of WHO size per cm^3; Diameter range (μm); Length range (μm); Mean and standard deviation Arithmetic Diameter (μm); Mean and standard deviation Arithmetic Length (μm); Geometric mean diameter (μm) and Geometric standard deviation; Geometric mean length (μm) and Geometric standard deviation.

2.3.2 Particles

Number of Particles evaluated microscopically; Mean Number of Particles/cm^3.

2.4 LUNG BURDEN

At each sacrifice time point and for each animal, the following parameters should be reported for each group:

2.4.1 Fibres

Number of fibres evaluated microscopically; Mean Total Fibres/lung; Mean WHO Fibres/lung; Mean number of fibres > 20 µm in length/lung; Mean number of fibres < 5 µm, 5-20 µm and of WHO size per lung, Diameter range (µm); Length range (µm); Mean and standard deviation Arithmetic Diameter (µm); Mean and standard deviation Arithmetic Length (µm); Geometric mean diameter (µm) and Geometric standard deviation; Geometric mean length (µm) and Geometric standard deviation. The mean lung burden calculated from the bivariate size distribution and density should also be reported.

2.4.2 Particles

Number of Particles evaluated microscopically; Mean Number of Particles/lung.

2.4.3 Summary Tables

In addition a summary table showing the following data for each time point should be provided:

Fibre type; study group; sacrifice time point (days); Mean total number particles/lung. Mean number and percent remaining of WHO fibres/lung; Mean number and percent remaining of fibres/lung in the following length categories: < 5 µm; 5-20 µm and > 20µm.

2.5 DATA ANALYSIS

2.5.1 Quality Control of Fibre Counting

As a quality criterion for the performance of the biopersistence tests, for fibres which do not split longitudinally (e.g. MMMF), the evolution over time of the sum of the length of all fibres present should be determined. This parameter should always decrease and never remain constant or increase during the experiment.

2.5.2 Fibre Clearance

The clearance of the number of fibres remaining in the lung > 20μm in length as a function of time following cessation of exposure should be analysed using non-linear regression techniques. The 100% value should be fixed at day one after cessation of exposure. The clearance of the number of WHO fibres and the number of fibres in the length categories < 5 μm and 5 - 20 μm should also be determined.

When analysing the results using non-linear exponential regression the following criteria should be used:

a) a single exponential can be used to fit the data if the regression explains at least 80 % of the variance.

Single exponential: Percent Fibre Remaining = a * exp (- b * Time)

b) otherwise a double exponential fit should be used to fit the data.

Double exponential:

Percent Fibre Remaining = a1 * exp (- b1 * Time) + a2 * exp (- b2 * Time)

The loss function should be weighted by the inverse of the variance (ref. (5)). This function should be fitted to the data starting on day 1 following the cessation of exposure.

The results should be presented graphically. In addition, the regression equations including all coefficients and error terms (including 95 % confidence intervals of the $T_{1/2}$) and the percentage of variation explained should be presented for each fibre size fraction. Tabulation of the individual values for each animal should be included as appendix to the report.

2.5.3 Clearance half-times:

2.5.3.1 Single exponential:

For each curve the clearance half-time corresponding to the coefficient b should be presented as follows:

$T_{1/2} = \ln 2 / b$

2.5.3.2 Double exponential:

For each curve two clearance half-times should be presented, one for the coefficient b_1 and another for coefficient b_2 as follows:

$T_{1/2}\text{-}1 = \ln 2 / b_1$ and $T_{1/2}\text{-}2 = \ln 2 / b_2$

These clearance half-times often correspond to a faster clearance phase followed by a slower clearance phase (ref. (6)). In order to provide an index of the complete clearance which includes both the fast and slower clearance half-times ($T_{1/2}$-1 and $T_{1/2}$-2), the combined weighted clearance times (W-$T_{1/2}$) should be determined and presented for each fibre size fraction by summing the product of each half-time weighted by its coefficient a_x as follows:

$$W\text{-}T_{1/2} = \left(\frac{a_1}{a_1 + a_2}\right) \times T_{1/2}\text{-}1 + \left(\frac{a_2}{a_1 + a_2}\right) \times T_{1/2}\text{-}2.$$

3. REPORTING

3.1 TEST REPORT

The final report should include but not be limited to:

- The identification of test material, either by name or code number.
- The composition and other appropriate characteristics of the test fibre.
- A description of the test rats, including strain, source, number, allocation, sex, body weight range, age, method of identification, housing, diet etc.
- A description of the exposure concentration, exposure regimen, and duration of the treatment periods.
- A description of all methods.
- A description of all results.
- All statistical results as described in Section on Data Analysis and Method above.
- Summary tables of clearance as described above.
- Other statistical treatment of results when appropriate.
- A summary and assessment of all adverse effects.
- Figures of body weights.
- Summary tables of antemortem clinical signs, mortality data, body weights and pulmonary lobes weights.
- Individual tables of body weights, lung burden data, pulmonary lobes weights and necropsy findings.
- Discussion of the results.
- Interpretation of the results.

4. REFERENCES

(1) WHO, World Health Organisation, Reference Methods For Measuring Airborne Man Made Mineral Fibres (MMMF), prepared by the WHO Regional Office for Europe, Copenhagen (1985).

(2) Bernstein, D.M., Morscheidt, Grimm, H.G., & Teichert, U., "The Evaluation of Soluble Fibres Using the Inhalation Biopersistence Model, A Nine Fibre Comparison", Inhalation Toxicology. 8, 345-385 (1996).

(3) Bernstein, D.M., Morscheidt, C., Tiesler, H., Grimm, H.G., Thevenaz, Ph. & Teichert, U., "Evaluation of the Biopersistence of Commercial and Experimental Fibres Following Inhalation", Inhalation Toxicology, 7 (7), 1029-1056 (1995).

(4) Bernstein, D.M., Mast, R., Anderson, R., Hesterberg, T.W., Musselman, R., Kamstrup, O., and Hadley, J., "An Experimental Approach To The Evaluation Of The Biopersistence Of Respirable Synthetic Fibres And Minerals.", Environmental Health Perspectives, 102, Supplement 5, 15-18 (1994).

(5) Neter, J., Wasserman, W. and Kutner, M.H., Applied Linear Statistical Models, Third Edition, Irwin, Inc., Homewood, Il. (1990).

(6) Stöber, W., McClellan, R.O., and Morrow, P., "Approaches to Modeling Disposition of Inhaled Particles and Fibres in the Lung." In: Toxicology of the Lung, 2nd ed., pp. 527-602, Gardner, D.E., Crapo, J.D., McClellan,R.O., eds., Raven Press, Ltd., New York, (1993).

(7) NF T 03-400 "Determination de la Biopersistence chez le rat – Essai par inhalation".

II.2 Biopersistence of Fibres. Intratracheal Instillation (ECB/TM/27 rev.7)

BXX. Biopersistence of Fibres. Intratracheal Instillation

1. METHOD

1.1 INTRODUCTION

See General Introduction Part B*(in Annex V of Dir. 67/548/EC)*.

1.2 DEFINITIONS

1. See General Introduction Part B in addition to the following:
2. *Biopersistence*: The ability of a material (fibre) to persist in the lung in spite of the lung's physiological clearance mechanisms and environmental conditions.
3. *Fibre* (also referred to as fibrous particles): An object with a length to width ratio (aspect ratio) of at least 3:1.
4. *WHO fibre*: A fibre with a length greater than 5 μm and a diameter less than 3 μm (reference. (1)).
5. *Particle* (also referred to as non-fibrous particles): An object with a length to width ratio of less than 3:1.

1.3 PRINCIPLE OF THE TEST METHOD

The objective of this method is to assess the in-vivo pulmonary biopersistence of instilled fibres and particles in the rat following repeated exposure for 4 days. This method is designed for the evaluation of synthetic mineral fibres, however, it can be applied with appropriate modification to organic and natural fibres.

This testing protocol is intended to be used as part of a tiered approach to the evaluation of fibres. This protocol evaluates the pulmonary biopersistence of fibres as a function of fibre length, however, it does not evaluate the effect of fibre diameter on human pulmonary deposition.

The specific use of the clearance of fibres longer than 20 μm in length in this protocol is designed to reflect the removal of fibres by dissolution and disintegration and is considered to be analogous in both humans and rats.

1.4 DESCRIPTION OF THE TEST METHOD

Laboratory rats are exposed by intratracheal instillation applied once each day on 4 consecutive days to well characterised fibre suspensions which have been optimised to be largely rat respirable. Following the end of the instillation period, subgroups of animals are sacrificed at pre-determined intervals and the lung burden determined by suitably validated extraction and measurement methods.

Measurements include characterisation of the number, bivariate size distribution and chemical composition of fibres (and particles) in the lung at predefined intervals following the cessation of the last exposure and determination of the time for removing 50 % of the fibres ($T_{1/2}$) which are longer than 20 μm. The $T_{1/2}$ of other length fractions: <5 μm, 5-20 μm WHO fibres should also be determined from the data.

1.4.1 Preparations

Healthy young adult animals are assigned to the control and treatment groups by randomisation by weight. The animals are kept in their cages for at least 5 days prior to the start of the study to allow for acclimatisation to the laboratory conditions.

The fibre under test is administered by intratracheal instillation. The method chosen for preparation and administration of the fibres should not contaminate the fibres and it should minimise alteration the fibre surface or the production of non fibrous dust (through excessive grinding or abrasion of the fibres).

1.4.2 Test conditions

1.4.2.1 Experimental animals

Rat, Fischer 344 or Wistar supplied specific pathogen free (SPF), virus antigen-free (VAF+) and maintained under optimum hygienic conditions (OHC). Upon receipt and at selected intervals throughout the study, a sentinel group of rats should be analysed for bacteriological and viral contamination. Another strain of laboratory rat may be used providing it is validated in comparison to the Fisher 344 or Wistar.

The animals should be maintained under barrier conditions throughout the study. The rats should be acclimated to the animal room used for housing during the test for at least 5 days after clinical health examination. The rats should be approximately 8-10 weeks old with a weight range of ± 20 % of the mean at the start of the acclimation period. The exact age and weight should be recorded as part of the study data.

1.4.2.2 Number and sex

At least 7 rats (either male or female) /group/sacrifice time point should be used. One sex only is used as no difference has been reported in the response to chronic fibre inhalation in male and female rats. In order to eliminate deviant values, the lung fibre content should be evaluated statistically to remove up to two extreme outliers if necessary. If additional end points are planned, the number should be increased by the number of animals scheduled to be killed before the completion of the study. For the control group, five animals / group / sacrifice time should be used.

1.4.2.3 Dose Level

1.4.2.3.1 CHOICE OF EXPOSURE CONCENTRATION

Animals should be exposed to a single concentration of the test substance. The size distribution of the instilled fibres should be similar to that used for inhalation biopersistence studies, if technically feasible. That is:

- A mean aspect ratio of at least 3:1,
- A rat respirable fibre diameter is preferred (Diameter as close as possible to 0.8 µm) with an upper limit in any case of 3 µm (95 % less than 3 µm). For each fibre the instillation procedure should be validated that no aggregates are formed in the suspensions or in the bronchi. If fibres with length L> 40 µm are present, extreme care should be taken to avoid aggregation in the airways,
- At least a 20 % of the WHO (L > 5 µm, D < 3 µm) fibres in suspension should have a length L > 20 µm and for this length fraction a geometric mean diameter as close as possible to 0.8 µm, if technically feasible.

It should be noted that diameters could be smaller than 0.8 µm if justified by the dimensions of the bulk fibre.

1.4.2.3.2 INSTILLATION DOSES

Two dose groups of total doses of 0.5 mg and 2 mg should be administered. The 0.5 mg dose simulates the approximate dose received following a five day inhalation exposure and minimises the possibility of acute inflammation and aggregation of fibres in the bronchi. The 2 mg dose is based upon the protocol developed by Bellmann and Muhle (4,5) for which there is an important database of studies.

The fibre samples should be suspended in 0.9 % NaCl in distilled water (isotonic solution, sterile, 308 mosm/l, pH = approx. 6).
The maximum volume instilled should be 0.4 ml/injection, if technically feasible.
The two dose groups should have a total mass of fibres injected of 0.5 mg and 2 mg, respectively.
The fibre suspensions should be prepared under sterile conditions from the bulk without altering their surface characteristics or contaminating the fibres.

1.4.2.3.3 DURATION AND FREQUENCY OF DOSING

The total exposure dose should be given to each group of animals in four (4) equal applications on consecutive days.

1.4.2.3.4 CONTROL FOR FIBRE AGGREGATES

Prior to the start of the study at least two animals should be dosed according to the above procedure with each fibre sample to be evaluated and the lungs examined by SEM (Scanning Electron microscopy) to confirm that no fibre aggregates (fibres that block ciliated airways) are formed following instillation. If aggregates are found, then either the diameter distribution should be reduced, the number of fibres longer

than 40 µm reduced or the dose administered reduced and the instillation repeated.

1.4.2.3.5 CONTROL GROUP DOSING

A control group is to be included. The treatment of the control animals will be performed under the same conditions as for the animals receiving the test fibres. All rats from this group will be dosed with 0.9 % NaCl in distilled water (isotonic solution, sterile, 308 mosm/l, pH = approx. 6).

1.4.2.4 Characterisation of the Test Article

The chemical composition of the fibre material supplied for testing at least to within 0.5 % and the density of the fibres should be provided.

1.4.2.5 Observation period

Sub-groups of animals should be sacrificed at a sufficient number of intervals after cessation of exposure so as to permit good definition of the clearance curves and calculation of the clearance half-times for each length fraction. The following sacrifice intervals have been used frequently in published biopersistence studies and are provided so as to permit cross comparison between studies:

Sacrifice intervals: 1 day, 2 days, 3 days, 14 days, 4 weeks, 3 months, 6 months, 12 months after the last instillation.

All studies should include at least sacrifice time intervals at 2 days, 14 days, 4 weeks and 3 months. Additional sacrifice intervals can be included as considered necessary to obtain further definition of the shape of the clearance curve. The background level/limit of detection for the fibres in the treated lungs should be determined based upon the control lungs. At each sacrifice, if an exposure group has a mean concentration of fibres >20 µm in length which is less than 5 % of the number found on day 2, the remaining animals in that group should be terminated without further analysis.

1.4.2.6 Laboratory Validation Fibre

Prior to starting studies on test fibres, laboratories with no previous experience in testing fibres should analyse a validation fibre using a similar exposure regime. All laboratories should analyse the validation fibre according to this protocol if no similar test has been carried out in the last 5 years. Fibres such as E-glass, MMVF21 or MMVF10a are recommended as the validation material. Optionally other fibres could be used as a validation material if sufficient data are available.

1.4.3 Procedure

1.4.3.1 Test Suspension preparation

In order to achieve the requirements stated in section 1.4.2.4, the bulk fibre used for instillation should be prepared or pre-selected by size to be respirable in the rodent. As a general guideline, for fibres of density $\rho \cong 2.4$, a geometric mean diameter as close to 0.8 µm as possible and a geometric mean length of approximately 15 µm will facilitate achieving those requirements.

If a pre-selection process is used or the bulk fibres used are produced by a non-commercial production method, a validation must be included in the study report which shows that the fibre has similar chemical and surface characteristics as compared to that produced commercially.

The suspensions of test fibre in saline should be prepared freshly each day immediately before the start of the instillations in order to minimise possible dissolution of the fibres. Suspensions should not be reused on subsequent days. The suspensions should be stirred continuously (e.g. magnetic stirrer) from the time of preparation through sampling and until all instillations of that fibre are completed.

The pH of each saline fibre suspension should be measured from the time of preparation until the instillation is complete. If the pH of the saline/fibre suspension increases above a pH of 9, then the saline/fibre suspension should be buffered using TRIS buffer.

1.4.3.2 Instillation

Before each treatment, the rats should be anaesthetised with a suitable anaesthetic. As soon as an animal is anaesthetised, it should be placed on its back on a slanted support (board) with its mouth kept open by retaining the upper incisor teeth with a tight rubber band.

A tracheal cannula should be inserted into the trachea of the rat. The diameter of the needle fitted to the syringe for collecting the suspension should be identical to the inside diameter of the cannula used for intratracheal instillation. The selected volume of the test material in suspension should be removed from a glass vessel (under constant stirring using a magnetic stirrer) using a syringe and gently injected into the trachea through the cannula.

All apparatus used in preparing and injecting the suspensions should be previously sterilised or disinfected.

1.4.3.3 Control Analysis of the Test Material - Vehicle Suspensions

1.4.3.3.1 SAMPLING INSTRUMENTS, SPECIFICATIONS AND CONTROL

All sampling of the suspension for the physical characterisation should be performed with a cannula fitted to a syringe. The diameter of the cannula fitted to the syringe should be identical to that of the cannula used for intratracheal instillation.

During the technical preparation of the experiment, for each fibre type, suspensions identical to that used for intratracheal instillation should be prepared. Sampling should be performed according to a technique identical to that planned for the experiment. The tip of the cannula should be observed under a binocular microscope to assure that there is no accumulation of fibres at the tip.

1.4.3.3.2 PHYSICAL CHARACTERISATION OF THE SUSPENSION

All samples described below should be taken within 30 seconds of vortex mixing.

Immediately after preparation of each suspension:

- One sample from the middle of the container should be taken for control of concentration and homogeneity in all groups treated with the test fibre by gravimetric evaluation.

- At least one sample (from the middle of the flask) should be taken for characterisation of fibre size by SEM in each group and for evaluation of the fibre concentration in the suspension (fibres/ml).

Immediately after end of instillation of each fibre type:

- One sample from the middle of the flask should be taken for control by gravimetry of conservation of suspension characteristics during the administration session.

1.4.3.3.3 MICROBIOLOGICAL CHARACTERISATION OF THE SUSPENSION

Immediately prior to each daily administration, 1 ml of each fibre suspension should be sampled with a sterile pipette and inoculated into Thioglycolate-Broth appropriate for supporting and growth of all major bacterial rat pathogens (aerobic, microaerophile, anaerobic).

1.4.3.4 General observations

1.4.3.4.1 MORTALITY

All animals should be observed for mortality/moribundity before the start and after the completion of each dosing and at least once on non-administration days.

1.4.3.4.2 CLINICAL SIGNS

Each animal should have a detailed clinical observation for signs of toxicity, including time of onset, intensity and duration:

- once during acclimatisation phase
- twice daily (once prior to the daily instillation) during the 4 treatment days
- once daily during the first two weeks after end of the dosing phase
- once weekly thereafter.

1.4.3.4.3 BODY WEIGHT

All animals should be weighed at least

- once at the beginning of the acclimatisation period,
- on the day of first instillation, prior to the start of administration,
- once weekly thereafter through week 12,
- once monthly thereafter.

1.4.3.4.4 GROSS NECROPSY

As a control of animal health, all animals in the study should be subjected to a full, detailed gross necropsy, which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. All animals should be anaesthetised and sacrificed by exsanguination. All gross necropsy findings in the lungs should be recorded.

1.4.3.4.5 ORGAN SAMPLING

Animals found dead or sacrificed moribund should be autopsied but their lungs not sampled for fibre analysis.

In a minimum of 5 animals/group per sacrifice:

- The lungs and the lower half of the trachea will be sampled.

Using a dissecting microscope:

- The lower half of the trachea and the main stem bronchi should be resected in one piece from the lung lobes, cleaned from remaining mediastinal tissues, weighed and immediately deep-frozen.

- The lung lobes should be weighed together and immediately deep-frozen.

The lungs should be frozen on dry ice and then stored at -20°C. All lungs should be freeze dried or critical point dried as quickly as possible thereafter and within two weeks following sacrifice to minimise possible fibre dissolution. Other appropriate methods can also be used if properly validated.

1.4.3.4.6 FURTHER PROCESSING OF LUNGS

The 7 animals allocated for sacrifice in each fibre group at each time-point are allocated for lung digestion and subsequent fibre analysis

Following drying of the rat pulmonary lobes, the dry lung weight should be determined. The dry tissue should then be digested using an appropriate method. For mineral fibres the preferred method is low temperature plasma ashing.

All tissue digestion methods should be validated prior to use. Using a standard addition procedure and a minimum of 3 previously unexposed rat lungs / dose group, doses of 0.05, 0.1 and 0.5 mg of a similar well characterised fibre should be injected and the lungs digested. The mean fibre number should be within ± 25 % of what was injected and the size distribution recovered should not be statistically different from that injected.

1.4.3.5 Fibre Evaluation and analysis

The following specifies the minimum analyses that should be performed on fibrous suspensions. If anomalies in the results occur, additional filters should be analysed if available.

1.4.3.5.1 GRAVIMETRIC DETERMINATION

The gravimetric concentration should be determined from each sample and expressed in mg/ml. These determinations should be used for assuring the uniformity of the instillation suspensions on a day to day basis.

1.4.3.5.2 FIBRE COUNT MEASUREMENT

Analyses should be performed by Scanning Electron Microscopy on the samples specified and expressed as total number of fibres/ml.

1.4.3.5.3 FIBRE SIZE DISTRIBUTION

Bivariate analysis of diameter and length should be determined by Scanning Electron Microscopy.

1.4.3.5.4 CHEMICAL ANALYSIS

One sample should be taken from the suspension for possible analysis.

1.4.3.6 Counting and Sizing Rules for Fibres and Particulates (for suspensions and lung fibres)

The general guidelines provided by the WHO/EURO (ref. (1)) are recommended with the following additional procedures for mineral fibres (due to the possibility of smaller diameters, additional procedures may be required for natural or organic fibres):

1.4.3.6.1 LENGTH AND DIAMETER

Sizing of length and diameters should be performed using a SEM at a magnification of at least 2000. All objects which seen at this magnification are to be counted. Fibres crossing the boundary of the field of view should be counted as follows. Fibres with only one end in the field are weighted as half of a fibre and fibres with neither of their ends in the field are not measured. Diameters of fibres which are seen at 2000 magnification should be measured at 'full screen' magnification (usually up to a magnification of 10,000). No lower or upper limit is to be imposed on either length or diameter. For bulk fibres with mean diameters below a few tenths of a micrometer, an initial magnification of at least 5000 should be used.

The length and diameter are to be recorded individually for each fibre measured so that the bivariate distribution can be determined. When sizing, an object is to be accepted as a fibre if the ratio of length to diameter was at least 3:1. All other objects are considered particles. There should be no truncation in the measurements. If fibre measurements are made using SEM photomicrographs or video prints where the magnification of the photo or print is at least twice that of the SEM screen, then an initial SEM magnification of at least 1000 is acceptable, providing fibres of 0.1 μm diameter can be resolved. When using photomicrographs or video prints, higher magnifications should be used for diameter measurement than for length measurement in order to ensure good precision.

1.4.3.6.2 STOPPING RULES

Enough fields of view are to be counted for evaluation so that at least a total of 0.15 mm^2 of the filter surface (for 25 mm diameter) is examined. Once this condition is fulfilled:

1. Fibres: The evaluation of fibres should be stopped when 400 WHO (L > 5 μm, D < 3 μm) fibres are counted/measured for each sample (lung/filter etc) analysed by SEM or a total of 1000 fibres and particles were recorded, or 1 mm^2 of the filter surface was examined, even if a total of 400 countable WHO fibres was not reached. Otherwise, the procedural variability and counting errors could result in a false estimate of the measure. The total number of fibres per filter should be determined by normalising the surface area counted to the total surface area of the filter.

2. Particles: The recording of particles can be stopped when a total of 100 particles are counted. If the size distribution of particles is measured, care should be taken in the lung samples to confirm by EDAX which particles are of the same composition as the fibres.

Other strategies for measurement (such as size selective analysis using a minimum of 100 fibres per category with at least 3 length categories) can be used providing that the method has been validated to produce similar results statistically in comparison to the above method.

2. DATA

2.1 ANIMAL DATA

Individual data should be provided. Additionally, all data should be summarised in tabular form showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons and the time of any death or humane kill, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the number of animals showing lesions the type of lesions and the percentage of animals displaying each type of lesion. In addition, the body weights and organ weights should be provided.

2.2 FIBRE CHEMISTRY

The chemical composition of the fibre material provided for testing to within at least 0.5 % and the density of the fibres tested should be presented

2.3 FIBRE SUSPENSIONS

The following parameters should be reported for the fibre instillation suspensions:

2.3.1 Fibres

Number of fibres evaluated microscopically; Mean and standard deviation gravimetric concentration (mg/ml); Mean Total Fibres/ml; Mean WHO Fibres/ml; Mean number of fibres > 20 μm in length/ml; Mean number of fibres < 5 μm, 5-20 μm and of WHO size per ml, Diameter range (μm); Length range (μm); Mean and standard deviation Arithmetic Diameter (μm); Mean and standard deviation Arithmetic Length (μm); Geometric mean diameter (μm) and Geometric standard deviation; Geometric mean length (μm) and Geometric standard deviation

In addition, the mean time from immersion of the fibres into saline to injection into the animal on each day of instillation should be provided. The individual values should be included in the raw data.

2.3.2 Particles

Number of Particles evaluated microscopically; Mean Number of Particles/ml.

2.4 LUNG BURDEN

At each sacrifice time point and for each animal, the following parameters should be reported for each group:

2.4.1 Fibres

Number of fibres evaluated microscopically; Mean Total Fibres/lung; Mean WHO Fibres/lung; Mean number of fibres > 20 μm in length/lung; Mean number of fibres < 5 μm, 5-20 μm and of WHO size per lung; Diameter range (μm); Length range (μm); Mean and standard deviation Arithmetic Diameter (μm); Mean and standard deviation Arithmetic Length (μm); Geometric mean diameter (μm) and Geometric standard deviation; Geometric mean length (μm) and Geometric standard deviation. The mean lung burden calculated from the bivariate size distribution and density should also be reported.

2.4.2 Particles

Number of Particles evaluated microscopically; Mean Number of Particles/lung.

2.4.3 Retained volume

The mean retained volume of fibres present in the lung should be calculated at the first sacrifice time point using the bivariate size distribution and the total number of fibres per lung.

2.4.4 Summary Tables

In addition a summary table showing the following data for each sacrifice time point should be provided:

Fibre type; study group; sacrifice time point (days); Mean total number particles/lung; Mean number and percent remaining of WHO fibres/lung; Mean number and percent remaining of fibres/lung in the following length categories: < 5μm; 5-20 μm and > 20 μm.

2.5 DATA ANALYSIS

2.5.1 Quality Control of Fibre Counting

As a quality criterion for the performance of the biopersistence tests, for fibres which do not split longitudinally (e.g. MMMF), the evolution over time of the sum of the length of all fibres present should be determined. This parameter should always decrease and never remain constant or increase during the experiment.

2.5.2 Fibre Clearance

The clearance of the number of fibres remaining in the lung > 20 μm in length as a function of time following cessation of exposure should be analysed using non-linear regression techniques. The 100 % value should be fixed at day two after the last instillation. The clearance of the number of WHO fibres and the number of fibres in the length categories < 5μm and 5-20 μm should also be determined.

When analysing the results using non-linear exponential regression the following criteria should be used:

a) a single exponential can be used to fit the data if the regression explains at least 80 % of the variance.

Single exponential:

$$\text{Percent Fibre Remaining} = a * \exp(-b * \text{Time})$$

b) otherwise a double exponential fit should be used to fit the data.

Double exponential:

$$\text{Percent Fibre Remaining} = a_1 * \exp(-b_1 * \text{Time}) + a_2 * \exp(-b_2 * \text{Time})$$

The loss function should be weighted by the inverse of the variance (ref. (2)). This function should be fitted to the data starting on day 2 following the last instillation.

The results should be presented graphically. In addition, the regression equations including all coefficients and error terms (including 95 % confidence intervals of the $T_{1/2}$) and the percentage of variation explained should be presented for each fibre size fraction. Tabulation of the individual values for each animal should be included as appendix to the report.

2.5.3 Clearance half-times:

2.5.3.1 Single exponential:

For each curve the clearance half-time corresponding to the coefficient b should be presented as follows:

$T_{1/2} = \ln 2 / b$

2.5.3.2 Double exponential:

For each curve two clearance half-times should be presented, one for the coefficient b_1 and another for coefficient b_2 as follows:

$T_{1/2}$-1 = ln 2 / b_1 and $T_{1/2}$-2 = ln 2 / b_2

These clearance half-times often correspond to a faster clearance phase followed by a slower clearance phase (ref. (3)). In order to provide an index of the complete clearance which includes both the fast and slower clearance half-times ($T_{1/2}$-1 and $T_{1/2}$-2), the combined weighted clearance times (W-$T_{1/2}$) should be determined and presented for each fibre size fraction by summing the product of each half-time weighted by its coefficient a_x as follows:

$$W\text{-}T_{1/2} = \left(\frac{a_1}{a_1 + a_2}\right) \times T_{1/2}\text{-}1 + \left(\frac{a_2}{a_1 + a_2}\right) \times T_{1/2}\text{-}2.$$

3 REPORTING

The final report should include but not be limited to:

- The identification of test materials either by name or code number.
- The composition and other appropriate characteristics of the test fibre.
- A description of the test rats, including strain, source, number, allocation, sex, body weight range, age, method of identification, housing, diet etc.
- A description of the doses, dose regimen and duration of the treatment periods.
- A description of all methods.
- A description of all results.
- All statistical results as described in Section on Data Analysis and Method above.
- Summary tables of clearance as described above.
- Other statistical treatment of results when appropriate.
- A summary and assessment of all adverse effects.
- Figures of body weights.
- Summary tables of antemortem clinical signs, mortality data, body weights and pulmonary lobes weights.
- Individual tables of body weights, lung burden data, pulmonary lobes weights and necropsy findings.
- Discussion of the results.
- Interpretation of the results.

4 REFERENCES

1. WHO, World Health Organization, Reference Methods For Measuring Airborne Man-Made Mineral Fibres (MMMF), prepared by the WHO Regional Office for Europe, Copenhagen (1985).

2. Neter, J., Wasserman, W. and Kutner, M.H., Applied Linear Statistical Models, Third Edition, Irwin, Inc., Homewood, Il. (1990).

3. Stöber, W., McClellan, R.O., and Morrow, P., "Approaches to Modeling Disposition of Inhaled Particles and Fibres in the Lung." In: Toxicology of the Lung, 2nd ed., pp. 527-602, Gardner, D.E., Crapo, J.D., McClellan,R.O., ... Raven Press, Ltd., New York, (1993)

4. Bellmann, B. and Muhle, M., "Investigation of the biodurability of woolastonite and xonotlite." Environ. Health Persp., 102, pp. 191-195 (1994).

5. Muhle, H., Koch, W., Bellmann, B. "Acute and subchronic effects of intratracheally instilled nickel containing particles in hamsters." In: Health Hazards and Biological Effects of Welding Fumes and Gases (Stein RM, Berlin A, Fletcher AC, Järvisalo J, eds). New York: Excerpta Medica, pp. 337-340 (1986).

II.3 Carcinogenicity of Synthetic Mineral Fibres after Intraperitoneal Injection in Rats (ECB/TM/18(97) rev. 1)

B.XX. Carcinogenicity of Synthetic Mineral Fibres after Intraperitoneal Injection in Rats

1 METHOD

1.1 INTRODUCTION

See General Introduction Part B*(in Annex V of Dir. 67/548/EC)*.

1.2 DEFINITIONS

1. See General Introduction Part B in addition to the following.
2. *Fibre* (also referred to as fibrous particles): An object with a length to width ratio (aspect ratio) of at least 3:1.
3. *WHO fibre*: A fibre with a length greater than 5 μm and a diameter less than 3 μm (ref. (1)).
4. *Particle* (also referred to as non-fibrous particles): An object with a length to width ratio of less than 3:1.

1.3 PRINCIPLE OF THE TEST METHOD

This method is designed to evaluate the tumorigenic response in the peritoneal cavity in rats exposed by intraperitoneal injection to well characterised synthetic mineral fibres. (refs. (2, 3)).

1.4 DESCRIPTION OF THE TEST METHOD

Laboratory rats are exposed by one or more intraperitoneal injections to well characterised fibre suspensions which have been optimised to consist of fibres which would be largely rat respirable. Following exposure, animals are maintained without further exposure until a total of 20 % survival occurs in the exposure group(s).

1.4.1 Preparations

Healthy young adult animals are assigned to the control and treatment groups by randomisation by weight. The animals are kept in their cages for at least 5 days prior to the start of the study to allow for acclimatisation to the laboratory conditions.

The fibre under test is administered by intraperitoneal injection of a suspension of fibres in saline. The method of fibre preparation is dependent on the physical chemical properties of the fibre. The method chosen should not contaminate the fibres and should minimise possible alteration of the fibre surface or the production of non-fibrous dust (through excessive grinding or abrasion of the fibres).

1.4.2 Test conditions

1.4.2.1 Experimental animals

Wistar rats ,supplied specific pathogen free (SPF), virus antigen-free (VAF+) and maintained under optimum hygienic conditions (OHC). Upon receipt and at selected intervals throughout the study, a sentinel group of rats should be analysed for bacteriological and viral contamination. Another strain of laboratory rat may be used providing it is validated in comparison to the Wistar.

The animals should be maintained under barrier conditions throughout the study. The rats should be acclimated to the animal room used for housing during the test for at least 5 days after clinical health examination. The rats should be approximately 8 - 10 weeks old with a weight range that should not exceed ± 20 % of the mean at the start of the acclimation period. The exact age and weight should be recorded as part of the study data.

1.4.2.2 Number and sex

At least 50 rats (females are preferred) /group, which are followed through their lifetime, should be assigned to each exposure and control group.

1.4.2.3 Choice of Dose Levels

Animals should be dosed using a single concentration of the test substance. The size distribution of the injected fibres should be similar to that used for chronic fibre inhalation studies, if technically feasible. That is:

- A mean aspect ratio of at least 3:1,
- A rat respirable diameter is preferred (Diameter as close as possible to 0.8 μm) with an upper limit in any case of 3 μm (95 % less than 3 μm). For each fibre the injection procedure should be validated that no aggregates are formed in the suspensions. If fibres with length L> 40 μm are present, extreme care should be taken to avoid aggregation,
- At least 20 % of the WHO (L > 5 μm, D < 3 μm) fibres in suspension should have a length L > 20 μm and for this length fraction a geometric mean diameter as close as possible to 0.8 μm, if technically feasible.

It should be noted that diameters could be smaller than 0.8 μm if justified by the dimensions of the bulk fibre.

1.4.2.3.1 INJECTION DOSES

For each fibre evaluated, a single dose of 1 x 10^9 WHO fibres per animal should be administered.

Optionally, if a dose-response relationship is desired, two additional doses may be administered of 1 x 10^7 and 1 x 10^8 WHO fibres per animal.

- The fibre samples should be suspended in 0.9 % NaCl in distilled water (isotonic solution, sterile, 308 mosm/l, pH = approx. 6).

- The maximum volume injected should be 5 ml/injection with a maximum of 25 mg of fibres in the 5 ml suspension, if technically feasible. If it is not possible to maintain a uniform suspension of 25 mg of fibres in the 5 ml, then lower suspension concentrations should be used with multiple injections. If more than 25 mg of fibre is necessary, then multiple injections should also be used. However, no more than 250 mg should be administered per animal.

The fibre suspensions should be prepared under sterile conditions from the bulk without altering their surface characteristics or contaminating the fibres. The time from immersion of the fibres into the saline until injection should be recorded for each animal. This time should be as short as possible especially for more soluble fibres.

1.4.2.3.2 DURATION AND FREQUENCY OF DOSING

The total dose should be given to each group of animals in one or more equal applications. If multiple applications are required, they should be administered at intervals every 7 days.

1.4.2.3.3 CONTROL GROUP DOSING

A control group is to be included. The treatment of the control animals should be performed under the same conditions as for the animals receiving the test fibres. All rats from this group should be dosed with 0.9 % NaCl in distilled water (isotonic solution, sterile, 308 mosm/l, pH = approx. 6).

1.4.2.4 Characterisation of the Test Article used for suspensions preparation

The chemical composition of the fibre material provided for testing to at least within 0.5 % and the density of these fibres should be provided.

1.4.3 Procedure

1.4.3.1 Test Suspension preparation

In order to achieve the requirements stated in section 1.4.2.4, the bulk fibre used for injection should be prepared or pre-selected by size that would be respirable in the rodent. As a general guideline, for fibres of density $\rho \cong 2.4$, a geometric mean diameter as close to 0.8 µm as possible and a geometric mean length of approximately 15 µm will facilitate achieving those requirements.

If a pre-selection process is used or the bulk fibres used are produced by a non-commercial production method, a validation must be included in the study report which shows that the fibre has similar chemical and surface characteristics as compared to that produced commercially.

The suspensions of test fibre in saline should be prepared freshly immediately before the start of the injections in order to minimise possible dissolution of the fibres. Suspensions should not be reused on subsequent days. The suspensions should be stirred continuously (e.g. magnetic stirrer) from the time of preparation through sampling and until all injections of that fibre are completed.

The pH of each saline fibre suspension should be measured from the time of preparation until the injection is complete. If the pH of the saline/fibre suspension increases above a pH of 9, then the saline/fibre suspension should be buffered using TRIS buffer.

1.4.3.2 Intraperitoneal Injection

The selected volume of the test material in suspension should be removed from a glass vessel (under constant stirring using a magnetic stirrer) using a syringe and gently injected through a needle inserted into the lower part of the abdominal cavity of the rat.

All apparatus used in preparing and injecting the suspensions should be previously sterilised or disinfected.

1.4.3.3 Control Analysis of the Test Material - Vehicle Suspensions

1.4.3.3.1 SAMPLING INSTRUMENTS, SPECIFICATIONS AND CONTROL

All sampling of the suspension for the physical characterisation should be performed with a needle fitted to a syringe of an identical size as that used for injection.

During the technical preparation of the experiment, for each fibre type, suspensions identical to that used for intraperitoneal injection should be prepared. Sampling should be performed according to a technique identical to that planned for the experiment. The tip of the needle should be observed under a binocular microscope to assure that there is no accumulation of fibres at the tip.

1.4.3.3.2 PHYSICAL CHARACTERISATION OF THE SUSPENSION

All samples described below should be taken within 30 seconds of vortex mixing.

Immediately after preparation of each suspension:

- At least one sample from the middle of the container should be taken for control of concentration and homogeneity in all groups treated with the test fibre by gravimetric evaluation.

- At least one sample (from the middle of the flask) should be taken for characterisation of fibre size by SEM in each group and for evaluation of the fibre concentration in the suspension (fibres/ml).

Immediately after the end of injection of each fibre type:

- One sample from the middle of the flask should be taken tor control by gravimetry of conservation of suspension characteristics during the administration session.

1.4.3.3.3 MICROBIOLOGICAL CHARACTERISATION OF THE SUSPENSION

Immediately prior to each daily administration, 1 ml of each fibre suspension should be sampled with a sterile pipette and inoculated into Thioglycolate-Broth appropriate for supporting and growth of all major bacterial rat pathogens (aerobic, microaerophile, anaerobic).

1.4.3.4 General observations

1.4.3.4.1 MORTALITY

All animals should be observed for mortality/moribundity at least once each day.

1.4.3.4.2 CLINICAL SIGNS

Each animal should have a detailed clinical observation for signs of toxicity, including time of onset, intensity and duration:

- at least once during acclimatisation phase
- at least once weekly during the study phase.

1.4.3.4.3 BODY WEIGHT

All animals should be weighed at least

- once at the beginning of acclimatisation period,
- on the day of first injection, prior to the injection,
- once weekly thereafter through week 13,
- once every second week thereafter.

1.4.3.4.4 GROSS NECROPSY

Full gross necropsy should be performed on all animals, including those which died during the experiment or were killed having been found in a moribund condition. Representative grossly visible lesions, tumours or lesions suspected of being tumours should be preserved. An attempt should be made to correlate gross observations with the microscopic findings.

All organs and tissues should be preserved for histopathological examination. This usually includes the following organs and tissues: pituitary, thyroid (including parathyroid), thymus, lungs (including trachea), heart, aorta, salivary glands, liver, spleen, kidneys, adrenals, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, uterus, urinary bladder, lymph nodes, pancreas, gonads, accessory genital organs, female mammary gland, skin, musculature, peripheral, nerve, spinal cord (cervical, thoracic, lumbar), sternum with bone marrow and femur (including joint) and eyes. Inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues; inflation of the lungs is essential for appropriate histopathological examination. Special attention should be given to describing all abnormal findings in the peritoneal cavity and diaphragm.

1.4.3.4.5 ORGAN SAMPLING AND ANALYSIS

When an obvious tumour is present, three blocks of tissue from separate tumour areas should be taken, processed and examined by a pathologist.

In all animals, the diaphragm including the falciform ligament, omentum, intestinal mesenteries plus gut segment, liver, spleen and pancreas should be removed using a dissecting stereomicroscope (ref. (4)). Other organs of the abdominal cavity may also be removed. Slides of these tissues should be examined by a pathologist. All abnormalities should be described and included in the report.

1.4.4 Study Monitoring

1.4.4.1 Fibre Evaluation and analysis

The following specifies the minimum analyses that should be performed on fibrous suspensions. If anomalies in the results occur, additional filters should be analysed if available.

1.4.4.1.1 GRAVIMETRIC DETERMINATION

The gravimetric concentration should be determined from each sample and expressed in mg/ml. These determinations should be used for assuring the uniformity of the injection suspensions on a day to day basis.

1.4.4.1.2 FIBRE COUNT MEASUREMENT

Analyses should be performed by Scanning Electron Microscopy on the samples specified and expressed as total number of fibres/ml.

1.4.4.1.3 FIBRE SIZE DISTRIBUTION

Bivariate analysis of diameter and length should be determined by Scanning Electron Microscopy.

1.4.4.1.4 CHEMICAL ANALYSIS

One sample should be taken from the suspension for possible analysis.

1.4.4.2 Counting and Sizing Rules for Fibres and Particulates

The general guidelines provided by the WHO/EURO (ref. (1)) are recommended with the following additional procedures for mineral fibres.

1.4.4.2.1 LENGTH AND DIAMETER

Sizing of length and diameters should be performed using a SEM at a magnification of at least 2000. All objects, which are seen at this magnification, are to be counted. Fibres crossing the boundary of the field of view should be counted as follows. Fibres with only one end in the field are weighted as half of a fibre and fibres with neither of their ends in the field are not measured. Diameters of fibres which are seen at 2000 magnification should be measured at 'full screen' magnification (usually up to a magnification of 10,000). No lower or upper limit is to be imposed on either length or diameter. For bulk fibres with mean diameters below a few tenths of a micrometer, an initial magnification of at least 5000 should be used. The length and diameter are to be recorded individually for each fibre measured so that the bivariate distribution can be determined. When sizing, an object is to be accepted as a fibre if the ratio of length to diameter was at least 3:1. All other objects are considered particles. There should be no truncation in the measurements. If fibre measurements are made using SEM photomicrographs or video prints where the magnification of the photo or print is at least twice that of the SEM screen, then an initial SEM magnification of at least 1000 is acceptable, providing fibres of 0.1 μm diameter can be resolved. When using photomicrographs or video prints, higher magnifications should be used for diameter measurement than for length measurement in order to ensure good precision.

1.4.4.2.2 STOPPING RULES

Enough fields of view are to be counted for evaluation so that at least a total of 0.15 mm^2 of the filter surface (for 25 mm diameter) is examined. Once this condition is fulfilled:

1. Fibres in saline suspension: The evaluation of fibres should be stopped when 1000 WHO ($L > 5$ μm, $D < 3$ μm) fibres are counted/measured for each sample (lung/filter etc) analysed by SEM or a total of 2500 fibres and particles were recorded, or 1 mm^2 of the filter surface was examined, even if a total of 1000 countable WHO fibres was not reached. Otherwise, the procedural variability and counting errors could result in a false estimate of the measure. The total number of fibres per filter should be determined by normalising the surface area counted to the total surface area of the filter.
2. Particles: The recording of particles can be stopped when a total of 30 particles are counted. If the size distribution of particles is measured, care should be taken in to confirm by EDAX which particles are of

the same composition as the fibres.

Other strategies for measurement (such as size selective analysis using a minimum of 100 fibres per category with at least 3 length categories) can be used providing that the method has been validated using comparable fibres to produce similar results statistically in comparison to the above method.

2 DATA

2.1 ANIMAL DATA

Individual data should be provided. Additionally all data should be summarised in tabular form showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons and the time of any death or humane kill, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the number of animals showing lesions, the type of lesions and the percentage of animals displaying each type of lesion. In addition, the body weights should be provided.

2.2 FIBRE CHEMISTRY

The chemical composition of the fibre material provided for testing to within at least 0.5 % and the density of the fibre tested should be presented.

2.3 FIBRE SUSPENSIONS

The following parameters should be reported for the fibre injection suspensions:

2.3.1 Fibres

Number of fibres evaluated microscopically; Mean and standard deviation gravimetric concentration (mg/ml); Mean Total Fibres/ml; Mean WHO Fibres/ml; Mean number of fibres > 20 μm in length/ml; Mean number of fibres < 5 μm, 5-20 μm and of WHO size per ml, Diameter range (μm); Length range (μm); Mean and standard deviation Arithmetic Diameter (μm); Mean and standard deviation Arithmetic Length (μm); Geometric mean diameter (μm) and Geometric standard deviation; Geometric mean length (μm) and Geometric standard deviation

In addition, the mean time from immersion of the fibres into saline to injection into the animal on each day of injection should be provided. The individual values should be included in the raw data.

2.3.2 Particles

Number of Particles evaluated microscopically; Mean Number of Particles/ml.

2.4 HISTOPATHOLOGY

Data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

2.5 DATA ANALYSIS

Results should be evaluated by an appropriate statistical method. Any recognised statistical method may be used.

3 REPORTING

3.1 TEST REPORT

The final report should include but not be limited to:

- The identification of test fibre, either by name or code number.
- The composition and other appropriate characteristics of the test fibres.
- A description of the test rats, including strain, source, number, allocation, sex, body weight range, age, method of identification, housing, diet etc.
- A description of the doses, dose regimen and duration the treatment periods.
- A description of all methods.
- A description of all results.
- All statistical results as described in Section on Data Analysis and Method above.
- Summary tables as described above.
- Other statistical treatment of results when appropriate.
- A summary and assessment of all adverse effects.
- Figures of body weights.
- Summary tables of antemortem clinical signs, mortality data and body weights
- Individual tables of body weights and necropsy findings.
- Tables of histopathological data.
- Discussion of the results.
- Interpretation of the results.

4 REFERENCES

(1) WHO, World Health Organisation, Reference Methods For Measuring Airborne Man Made Mineral Fibres (MMMF), prepared by the WHO Regional Office for Europe, Copenhagen (1985).

(2) Pott, F. et al., Beurteilung der kanzerogentiät inhalierbarer fasern. In: Faserförmige Stäube. Vorschriften, Wirkungen, Messung, Minderung, Tagung Fulda, 6-9 September 1993, Kommission Reinhaltung der Luft im VDI und DIN. (VDI-Berichte 1075) Düsseldorf, VDI-Verl. 1993, pp. 17-77.

(3)Summary Report of IInd MMF Workshop, Paris, 16 September 1994, International Cooperative Research Programme on Assessment of MMFs Toxicity, Margaux Orange Organisation, 75005 Paris, publisher.

(4) Moalli PA, Macdonald JL. Goodglick LA and Kane A. Acute injury and regeneration of the mesothelium in response to asbestos fibres, Am. J. Path. 128: 426-445 (1987).

II.4 Chronic Inhalation Toxicity of Synthetic Mineral Fibres in Rats (ECB/TM/17(97) rev.2)

B.XX. Chronic Inhalation Toxicity of Synthetic Mineral Fibres in Rats

1 METHOD

1.1 INTRODUCTION

See General Introduction Part B *(in Annex V to Dir 67/548/EC)*.

1.2 DEFINITIONS

1. See General Introduction Part B in addition to the following.
2. *Fibre* (also referred to as fibrous particles): An object with a length to width ratio (aspect ratio) of at least 3:1.
3. *WHO fibre*: A fibre with a length greater than 5 μm and a diameter less than 3 μm (reference (1)).
4. *Particle* (also referred to as non-fibrous particles): An object with a length to width ratio of less than 3:1.

1.3 PRINCIPLE OF THE TEST METHOD

This method is designed to assess the potential toxic response in rats to the inhalation of synthetic mineral fibres when exposed for 2 years. This method is based on the fact that rats exposed by inhalation to sufficiently durable long rat respirable fibres can exhibit pulmonary responses that include fibrosis, adenocarcinoma and mesothelioma. This method is also designed to quantify the fibre lung burden by size fraction in order to associate response with dose.

1.4 DESCRIPTION OF THE TEST METHOD

Laboratory rats are exposed by inhalation for 6 hours/day, 5 days/week for 104 consecutive weeks to well characterised fibre test atmospheres which have been optimised to be largely rat respirable. Following termination of the exposure phase, animals are maintained without further exposure until 20 % survival occurs in the test fibre exposure group. During and after exposure to the test fibre, the experimental animals are observed daily to detect signs of toxicity. At pre-determined intervals, subgroups of animals are sacrificed, the respiratory tract including pleural cavity evaluated for toxicity and the lung burden determined by suitably validated extraction and measurement methods (reference (2)).

1.4.1 Preparations

Healthy young adult animals are assigned to the control and treatment groups by randomisation by weight. The animals are kept in their cages for at least 5 days prior to the start of the study to allow for acclimatisation to the laboratory conditions. During this period, the animals should be placed in the nose-only restraint tubes on the exposure system for up to 6 hours/day for at least 4 days and exposed to filtered air at similar conditions that will be used during the study.

The fibre under test is administered by inhalation of an atmosphere of aerosolised fibres. The method of aerosolisation is dependent on the physical chemical properties of the fibre. The method chosen should not contaminate the fibres, and it should minimise possible alteration of the fibre surface or the production of non-fibrous dust (through excessive grinding or abrasion of the fibres).

1.4.2 Test conditions

1.4.2.1 Experimental animals

Wistar rats supplied specific pathogen free (SPF), virus antigen-free (VAF+) and maintained under optimum hygienic conditions (OHC) are preferred. Upon receipt and at selected intervals throughout the study, a sentinel group of rats should be analysed for bacteriological and viral contamination. Another strain of laboratory rat may be used providing it is validated in comparison to the Wistar.

The animals should be maintained under barrier conditions throughout the study. The rats should be acclimated to the animal room used for housing during the test for at least 5 days after clinical health examination. The rats should be approximately 8 - 10 weeks old with a weight range that should not exceed ± 20 % of the mean at the start of the acclimation period. The exact age and weight should be recorded as part of the study data.

1.4.2.2 Number and sex

At least 100 rats (males are preferred) /group, which are followed through their lifetime, should be assigned to each exposure and control group. In addition to the above animals, an additional 5 rats/group/time points should be for animals scheduled for interim sacrifices.

1.4.2.3 Animal room and housing during non-exposure periods

Animals should be housed in either stainless steel wire or polycarbonate cages. During the exposure period, cages for housing when the animals are not being exposed should be arranged in such a way that possible effects due to cross contamination of fibres from one group to another are minimised. If 2 or more test fibres are included in the study, each fibre group should be sufficiently isolated to minimise cross contamination between groups.

1.4.2.4 Choice of Exposure Concentrations (Dose Levels)

Animals should be exposed to up to three concentrations of the test substance. The fibre exposure aerosol should be prepared to be largely rat respirable and have:

- A mean aspect ratio of at least 3:1,

- The exposure concentrations stated below refer to the number of fibres with geometric mean length greater than 20 μm. The geometric mean diameter of those fibres longer than 20 μm should be as close to 0.8 μm as possible, for fibres with a density $\rho \cong 2.4$, if technically feasible For fibres with densities different from this the corresponding GMD should be determined. (Note: The GMD varies as the square root of the density for a constant median aerodynamic diameter).
- The gravimetric mean concentration of those fibres, which are 0.8 μm or less, should not exceed 40 mg/m^3 in the exposure aerosol, if technically feasible.

- As an upper limit, the gravimetric concentration of all particles (fibrous and non-fibrous) in the test atmosphere should not exceed 60 mg/m^3, if technically feasible.

The aerosol concentration to which the animals are exposed should be:
- At least 100 fibres/cm^3 longer than 20 μm in length with a GMD as close as possible to 0.8 μm.

As an option, multiple doses can be used to evaluate dose-response relationships. In this case the following additional doses are recommended:

1. 30 fibres/cm^3 longer than 20 μm in length with a GMD as close as possible to 0.8 μm.
2. 10 fibres/cm^3 longer than 20 μm in length with a GMD as close as possible to 0.8 μm.

A control group of animals exposed to filtered air only is to be included. The treatment of the control animals should be performed under the same conditions as for the animals receiving the test fibres.

It should be noted that diameters could be smaller than 0.8 μm if justified by the dimensions of the bulk fibre.

1.4.2.5 Characterisation of the Test Article used for aerosol generation

The chemical composition of the fibre material provided for testing to at least within 0.5 % and the density of these fibres should be provided.

1.4.2.6 Duration and Frequency of Exposure

Each group of animals should be exposed for 6 hours/day, 5 days/week for 104 consecutive weeks.

1.4.2.7 Observation period

Sub-groups of animals should be sacrificed:
- Following 90 days of exposure (Following study day 90)
- Following 360 days of exposure (Following study day 360)
- Following termination of exposure (Following study day 720)

All other animals are observed as described below and maintained until 20 % survival occurs in the exposure group at which time all remaining animals are sacrificed.

1.4.2.8 Laboratory Validation Fibre

Prior to starting studies on test fibres, laboratories with no previous experience in testing fibres should analyse a validation fibre by performing either a similar exposure regime as described herein or a 5-day inhalation biopersistence study. All laboratories should analyse the validation fibre according to either protocol if no similar test has been carried out in the last 5 years. Fibres such as E-glass, MMVF21 or MMVF 10a are recommended as the validation material. Optionally other fibres could be used as validation material if sufficient data are available.

1.4.3 Procedure

1.4.3.1 Test Article Preparation

In order to achieve the requirements stated in section 1.4.2.4, the bulk fibre used for aerosol generation should be prepared or pre-selected by size to be respirable in the rodent. As a general guideline, for fibres of density $\rho \cong 2.4$, a geometric mean diameter as close to 0.8 µm as possible and a geometric mean length of approximately 15 µm will facilitate achieving those requirements.

If a pre-selection process is used or the bulk fibres used are produced by a non-commercial production method, a validation must be included in the study report which shows that the fibre has similar chemical and surface characteristics as compared to that produced commercially.

1.4.3.2 Fibre Aerosol Generation system

The fibre aerosol generation system must be capable of producing the required aerosol concentration of fibres as described above continuously for a period of 6 hours/day without contaminating the fibres, altering the fibre surface or producing non-fibrous dust (through grinding or abrasion of the fibres). A suitable charge neutraliser (e.g. Ni63) should be placed immediately following the aerosol generator to assure that the fibres are discharged to Boltzmann equilibrium

The system should be able to produce the required aerosol exposure with a mean uniformity of plus or minus 15 % based upon gravimetric measurements of aerosol concentration.

1.4.3.3 Inhalation Exposure System

It is recommended that the flow-past nose-only exposure system (references (3), (4)) be used. Other nose-only exposure systems may be used if they are validated with respect to equivalent or greater fibre deposition in the lung of fibres > 20 µm in length as compared with the flow-past system.

The testing facility must provide documentation showing that the uniformity of fibre concentrations at the top, middle and bottom level of the exposure system is within ± 15 %.

1.4.3.4 General observations

1.4.3.4.1 MORTALITY

All animals should be observed for mortality/moribundity before the start and after the completion of each exposure and at least once on non-administration days.

1.4.3.4.2 CLINICAL SIGNS

Each animal should have a detailed clinical observation for signs of toxicity, including time of onset, intensity and duration:

- at least once during acclimatisation phase
- at least once weekly during the exposure and recovery phase.

Careful observations should be made to detect the onset and progression of all toxic effects as well as to minimise loss due to disease, autolysis or cannibalism.

1.4.3.4.3 BODY WEIGHT

All animals should be weighed at least

- once at the beginning of acclimatisation period,
- on the day of first exposure, prior to the start of exposure,
- once weekly thereafter through week 13,
- once every second week thereafter.

1.4.3.4.4 GROSS NECROPSY

Full gross necropsy should be performed on all animals, including those which died during the experiment or were killed having been found in a moribund condition. All grossly visible lesions, tumours or lesions suspected of being tumours should be preserved. An attempt should be made to correlate gross observations with the microscopic findings.

Inflation of lungs with a fixative is the optimal way to preserve this tissue and is essential for appropriate histopathological examination. The entire respiratory tract should be studied, including nose, pharynx, larynx and pleura.

All other organs and tissues should be preserved for possible histopathological examination. This usually includes the following organs and tissues: brain (medulla/pons, cerebellar cortex, cerebral cortex), pituitary, thyroid (including parathyroid), thymus, heart, aorta, salivary glands, liver, spleen, kidneys, adrenals, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, uterus, urinary bladder, lymph nodes, pancreas, gonads, accessory genital organs, female mammary gland, skin,

musculature, peripheral, nerve, spinal cord (cervical, thoracic, lumbar), sternum with bone marrow and femur (including joint) and eyes.

1.4.3.4.5 ORGAN SAMPLING AND ANALYSIS

At interim sacrifices, a minimum of 5 animals/group per sacrifice should be sampled and the lungs analysed as described below for fibre lung burden and histopathological analysis. At the final (terminal) sacrifice and on animals found moribund or dead, only histopathological analysis should be performed with the whole lung prepared as described below for histopathological examination.

Upon removal, all lungs should be cleaned from attached mediastinal tissues, including mediastinal lymph nodes and then weighed.

At interim sacrifices, if no relevant macroscopic findings are observed under the dissecting microscope in the right caudal lobe and in the accessory lobe:

the bronchi leading to these lobes should be ligated; the lobes should be removed, weighed separately and deep frozen on dry ice and stored at -20°C without fixation for subsequent lung burden analysis.

In case of occurrence of a relevant lesion in one or both of these lobes, the lung lobes with lesions present should not be used for the determination of fibre lung burden but should be fixed as are the remaining lobes for histopathological examination.

Other lobes (for histopathological examination): The lungs (with the few remaining mediastinal lymph nodes) should then be inflated with an appropriate fixative by instillation under a pressure of 30 cm H_20 and fixed by immersion for a minimum of 2 hours in the fixative. After approximately 48 hours in fixative, the main bronchus leading to the right median lobe should be cut off. This lobe should be transferred to 95 % ethanol, and forwarded within a short delay to the histotechnological processing.

1.4.3.4.5.1 Lung Burden

The lung lobes that were stored at -20°C, should be freeze dried or critical point dried as quickly as possible after collection and within two weeks of sacrifice to minimise possible fibre dissolution. Other appropriate methods can also be used. These lobes are to be allocated for lung digestion and subsequent fibre analysis.

Following drying of these lobes, the dry lung weight should be determined. The dry tissue should then be digested using an appropriate method. For mineral fibres the preferred method is low temperature plasma ashing.

All tissue digestion methods should be validated prior to use. Using a standard addition procedure and a minimum of 3 previously unexposed rat lungs / dose group, doses of 0.05, 0.1 and 0.5 mg of a similar well characterised fibre should be injected and the lungs digested. The mean fibre number should be within ± 25 % of what was injected and the size distribution recovered should not be statistically different from that injected.

1.4.3.4.5.2 Histopathological examination

The left pulmonary lobe and the right cranial lobe of the lung (if indicated, additional parts of the lungs may also be processed), the trachea and relevant gross lesions in any organ/tissue from all animals should be embedded in paraffin, cut at a nominal thickness of 4 micrometers and stained with hematoxylin and eosin. Other stains can be used if appropriate and multiple sections are recommended. An additional set of slides from the lung should be stained with a collagen specific stain.

All slides from all animals should be examined by a pathologist. All abnormalities should be described and included in the report. Evaluations of lung tissue should include assessment of collagen deposition at the broncho-alveolar junction, pulmonary fibrosis, interstitial fibrosis, peribronchiolar lesions and pleural changes. The severity of fibrotic response and the area of the lung showing fibrosis should be quantified.

1.4.4 Study Monitoring

1.4.4.1 Exposure system monitoring

- Airflow rate (monitored continuously and recorded at least once per day)
- Oxygen concentration (at least once per day). The oxygen concentration in the vicinity of the animal's nose should be maintained at a level of at least 19.5 %. If the flow-past nose-only exposure system is used and the air flow supplied to each animal is at least 1 l/min, then it is not necessary to measure the oxygen concentration.
- Temperature & humidity of the air supply (at least once per day). The temperature should be maintained at 22 ± 2 °C. To achieve the fibre aerosol exposures, it is recognised that the supply air to the generator can be dry and as such no lower limit is placed upon humidity. Review of the air control groups from a series of chronic studies has shown that there is no adverse effect from such low humidity.

1.4.4.2 Exposure atmosphere monitoring and analysis

All sampling for measurement of the aerosol exposure concentration and size distribution should be performed near where the animal's nose would be in the exposure system.

For the air control group, the sampling duration should be as long as possible (approximately 3 to 5 hours) in order to permit the assessment of the absence of contamination.

Sufficient monitoring of the exposure atmosphere should be performed during the pre-study phase in order to assure that the required fibre aerosol concentrations and uniformity are achieved during the study.

If a flow past exposure system is not used, sampling should be performed using methodology designed to minimise anisokinetic sampling errors.

The analyses specified below should be considered as the minimum analyses that should be performed. If anomalies in the results occur, additional filters should be analysed if available.

1.4.4.2.1 GRAVIMETRIC (mg/m^3)

If gravimetric concentration is used for monitoring of the aerosol fibre number, aerosol mass monitoring should be performed daily for a duration representative of the daily concentration. Daily sampling should be performed for at least 2 hours per day with each individual sample at least one hour. The gravimetric concentration should be determined from each filter sampled and expressed in mg/m^3.

1.4.4.2.2 FIBRE AND PARTICULATE NUMBER (fibres/cm^3) AND BIVARIATE SIZE DISTRIBUTION (µm) BY SCANNING ELECTRON MICROSCOPY (SEM)

These should be sampled at least once per day. These samples should be taken in timely coincidence with the gravimetric sampling with sample duration dependent upon fibre type (usually less than 30 minutes). One filter per day during the first week and two samples/week thereafter should be analysed for fibre and particulate number, with the remaining filters used if anomalies are found. Bivariate analysis of diameter and length should be determined at least twice weekly with the additional filters used if anomalies are found. Fibre concentration should be expressed as total number of fibres/cm^3 and the number of fibres/cm^3 with length > 20 µm, 5-20 µm, < 5 µm and WHO fibres and the number of particles/cm^3.

1.4.4.2.3 CHEMICAL ANALYSIS

One filter sample should be taken for possible analysis.

1.4.4.3 Counting and Sizing Rules (for aerosol and lung fibres

The general guidelines provided by the WHO/EURO (reference (1)) are recommended with the following additional procedures for mineral fibres.

1.4.4.3.1 1.4.4.3.1 LENGTH AND DIAMETER

Sizing of length and diameters should be performed using a SEM at a magnification of at least 2000. All objects, which are seen at this magnification, are to be counted. Fibres crossing the boundary of the field of view should be counted as follows. Fibres with only one end in the field are weighted as half of a fibre and fibres with neither of their ends in the field are not measured. Diameters of fibres which are seen at 2000 magnification should be measured at 'full screen' magnification (usually up to a magnification of 10,000). No lower or upper limit is to be imposed on either length or diameter. For bulk fibres with mean diameters below a few tenths of a micrometer, an initial magnification of at least 5000 should be used. The length and diameter are to be recorded individually for each fibre measured so that the bivariate distribution can be determined. When sizing, an object is to be accepted as a fibre if the ratio of length to diameter was at least 3:1. All other objects are considered particles. There should be no truncation in the measurements. If fibre measurements are made using SEM photomicrographs or video prints where the magnification of the photo or print is at least twice that of the SEM screen, then an initial SEM magnification of at least 1000 is acceptable, providing fibres of 0.1 µm diameter can be resolved. When using photomicrographs or video prints, higher magnifications should be used for diameter measurement than for length measurement in order to assure good precision.

1.4.4.3.2 STOPPING RULES

Enough fields of view are to be counted for evaluation so that at least a total of 0.15 mm^2 of the filter surface (for 25 mm diameter) is examined. Once this condition is fulfilled:

1. Fibres: The evaluation of fibres should be stopped when 400 WHO (L > 5 µm, D < 3 µm) fibres are counted/measured for each sample (lung, filter, etc.) analysed by SEM, or a total of 1000 fibres and non-fibrous particles were recorded, or 1 mm^2 of the filter surface was examined, even if a total of 400 countable WHO fibres was not reached. Otherwise, the procedural variability and counting errors could result in a false estimate of the measure. The total number of fibres per filter should be determined by normalising the surface area counted to the total surface area of the filter.
2. Particles: The recording of particles can be stopped when a total of 30 particles are counted. If the size distribution of particles is measured, care should be taken in the lung samples to confirm by EDAX which particles are of the same composition as the fibres.

Other strategies for measurement (such as size selective analysis using a minimum of 100 fibres per category with at least 3 length categories) can be used providing that the method has been validated using comparable fibres to produce similar results statistically in comparison to the above method.

2 DATA

2.1 ANIMAL DATA

Individual data should be provided. Additionally all data should be summarised in tabular form showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons and the time of any death or humane kill, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the number of animals showing lesions, the type of lesions and the percentage of animals displaying each type of lesion. In addition, the body weights and lung weights should be provided.

2.2 FIBRE CHEMISTRY

The chemical composition of the fibre material provided for testing to within at least 0.5 % and the density of the fibre tested should be presented.

2.3 EXPOSURE AEROSOL

The following parameters should be reported for the exposure aerosol:

2.3.1 Fibres

Number of fibres evaluated microscopically; Mean and standard deviation gravimetric concentration (mg/m^3); Mean Total Fibres/cm^3; Mean WHO Fibres/cm^3; Mean number of fibres > 20 µm in length/cm^3;

Mean number of fibres < 5 μm, 5-20 μm and of WHO size per cm^3; Diameter range (μm); Length range (μm); Mean and standard deviation Arithmetic Diameter (μm); Mean and standard deviation Arithmetic Length (μm); Geometric mean diameter (μm) and Geometric standard deviation; Geometric mean length (μm) and Geometric standard deviation.

2.3.2 Particles

Number of Particles evaluated microscopically; Mean Number of Particles/cm^3.

2.4 LUNG BURDEN

At each sacrifice time point and for each animal, the following parameters should be reported for each group:

2.4.1 Fibres

Number of fibres evaluated microscopically; Mean Total Fibres/lung; Mean WHO Fibres/lung; Mean number of fibres > 20 μm in length/lung; Mean number of fibres < 5 μm, 5-20 μm and of WHO size per lung, Diameter range (μm); Length range (μm); Mean and standard deviation Arithmetic Diameter (μm); Mean and standard deviation Arithmetic Length (μm); Geometric mean diameter (μm) and Geometric standard deviation; Geometric mean length (μm) and Geometric standard deviation.

2.4.2 Particles

Number of Particles evaluated microscopically; Mean Number of Particles/lung.

2.4.3 Summary Tables

In addition a summary table showing the following data for each time point should be provided:

Fibre type; study group; sacrifice time point (days); Mean total number particles/lung. Mean number and percent remaining of WHO fibres/lung; Mean number and percent remaining of fibres/lung in the following length categories: < 5 μm; 5-20 μm and > 20μm.

2.5 HISTOPATHOLOGY

Data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

In addition, the amount of collagen deposition at the broncho-alveolar junctions should be presented as a grade from 0-5 (see Annex). The chronic pulmonary changes and fibrosis should also be evaluated

according to a grading system such as that presented by McConnell et al. (ref. (5)). The severity of fibrotic response and the area of the lung showing fibrosis should be presented.

2.6 DATA ANALYSIS

Results should be evaluated by an appropriate statistical method. Any recognised statistical method may be used.

3 REPORTING

3.1 TEST REPORT

The final report should include but not be limited to:

- The identification of test materials, either by name or code number.
- The concentration, composition and other appropriate characteristics of the test fibre.
- A description of the test rats, including strain, source, number, allocation, sex, body weight range, age, method of identification, housing, diet etc.
- A description of the exposure concentrations, exposure regimen, and duration of the treatment periods.
- A description of all methods.
- A description of all results.
- All statistical results as described in Section on Data Analysis and Method above.
- Summary tables as described above.
- Other statistical treatment of results when appropriate.
- A summary and assessment of all adverse effects.
- Figures of body weights.
- Summary tables of antemortem clinical signs, mortality data, body weights and pulmonary lobes weights.
- Individual tables of body weights, lung burden data, pulmonary lobes weights and necropsy findings.
- Tables of histopathological data
- Discussion of the results.
- Interpretation of the results.

4 REFERENCES

(1) WHO, World Health Organisation, Reference Methods For Measuring Airborne Man Made Mineral Fibres (MMMF), prepared by the WHO Regional Office for Europe, Copenhagen (1985).

(2) Bernstein, D.M., Thevenaz, P., Fleissner, Anderson, R., H., Hesterberg and Mast, R., "Evaluation of the Oncogenic Potential of Man-Made Vitreous Fibres: The Inhalation Model", Ann. Occup. Hyg., Vol. 39 (5), 661-672 (1995).

(3) Cannon, W.C., Blanton, E.F., and McConald, K.E. The flow-past chamber: an improved nose-only exposure system for rodents. Am. Ind. Hyg. Assoc. J., 44(12) pp. 923-928 (1983).

(4) Bernstein, D.M., Mast, R., Anderson, R., Hesterberg, T.W., Musselman, R., Kamstrup, O., and Hadley, J., "An Experimental Approach To The Evaluation Of The Biopersistence Of Respirable Synthetic Fibers And Minerals.", Environmental Health Perspectives, 102, Supplement 5, 15-18 (1994).

(5) McConnell E.E., Wagner, J.C., Skidmore, J.W. and Moore, J.A. (1984) A comparative study of the fibrogenic and carcinogenic effects of UICC Canadian chrysotile B asbestos and glass microfibre (JM 100). In Biological Effects of Man-made Mineral Fibres, pp. 234-252. Proceed. WHO/IARC Conference, Copenhagen, 20-22 April, 1982, Vol. 2, World Health Organization, Geneva, Switzerland.

ANNEX

Histopathological Scoring Systems

EPS scoring system for Collagen Deposition at the Bronchiolar Alveolar Junction (1)

The histologic change "collagen deposition at the bronchiolar-alveolar junction" is described according to distribution, severity, and morphologic character.

Distribution is described as focal, multi-focal, or diffuse.

Severity scores are assigned as follows:

Grade 0 is characterized as no remarkable changes (refers to a normal lung).

Grade 1 is characterized by minimal, just detectable, very few, very small foci of collagen deposition. A lesion of this severity was not considered to be sufficient to apply grade 4 in the Wagner-scoring system.

Grade 2 is characterized by slight, fairly easily detected, few, small foci of collagen deposition. Lesions of this severity represented the lowest level of grade 4 in the Wagner-scoring system.

Grade 3 is characterized by moderate, easily detected foci of collagen deposition in considerably enlarged areas at the bronchiolar-alveolar junction. Lesions of this severity also represented grade 4 in the Wagner-scoring system.

Grade 4 is characterized by marked, obvious or extensive foci of collagen deposition extending from the bronchiolar-alveolar junction into the interstitium of more peripheral parts of the lung parenchyma. Lesions of this severity also represented grade 4 in the Wagner-scoring system.

Grade 5 is characterized by severe, widespread collagen deposition with consolidation at the bronchiolar-alveolar junction, sometimes with interlobular linking. Lesions of this severity represented grade 4 to 5 in the Wagner-scoring system.

Wagner-scoring system (2)

The histologic scoring system according to the criteria given by Wagner et al. (2), is also used to describe collagen deposition. In this scoring system a pulmonary change, grade 1.0 is considered normal, grades 2.0 and 3.0 are characterized by cellular changes (alveolar macrophages, microgranulomas, bronchiolization), and grades 4.0 to 8.0 represent the former lesions plus increasing distribution and degrees of fibrosis. The degree of fibrosis may range from minimal to moderate, but is consistent with grade 4.0 as long as no interlobular linking of the aforementioned lesions occurs. Grades 2.0 and 3.0 are considered potentially reversible while grades 4.0 to 8.0 are thought to be non-reversible.

REFERENCES

(1) Jorg Chevalier, Experimental Pathology Services AG, CH-4132 Muttenz (Basel), Switzerland. Personal communication.

(2) Wagner, J.C., et al., The effects of the inhalation of asbestos in rats, British journal of Cancer, 29, 252-269 (1974).

II.5 Sub-chronic Inhalation Toxicity of Synthetic Mineral Fibres in Rats (ECB/TM/16(97) rev. 1)

B.XX. Sub-chronic Inhalation Toxicity of Synthetic Mineral Fibres in Rats

1 METHOD

1.1 INTRODUCTION

See General Introduction Part B*(in Annex V to Dir 67/548/EC)*.

1.2 DEFINITIONS

1. See General Introduction Part B in addition to the following.
2. *Fibre* (also referred to as fibrous particles): An object with a length to width ratio (aspect ratio) of at least 3:1.
3. *WHO fibre*: A fibre with a length greater than 5 μm and a diameter less than 3 μm (ref. (1)).
4. *Particle* (also referred to as non-fibrous particles): An object with a length to width ratio of less than 3:1.

1.3 PRINCIPLE OF THE TEST METHOD

This method is designed to assess the potential for synthetic mineral fibres to produce cellular and pathological changes in the lungs of rats following exposure by inhalation to several doses for 13 weeks. Such early changes are considered as possible precursors to chronic irreversible effects such as interstitial fibrosis and tumours. The study is also designed to assess the potential for reversibility of any such changes and to permit association of responses with fibre dose in the lung and the influence of fibre length.

1.4 DESCRIPTION OF THE TEST METHOD

Groups of laboratory rats are exposed by inhalation for 6 hours/day, 5 days/week for 13 consecutive weeks to well characterised fibre test atmospheres which have been optimised to be largely rat respirable. Following termination of the exposure phase, animals are maintained without further exposure. At pre-determined intervals, subgroups of animals are sacrificed, the pulmonary response evaluated as defined herein and the lung burden determined by suitably validated extraction and measurement methods (ref. (2)).

1.4.1 Preparations

Healthy young adult animals are assigned to the control and treatment groups by randomisation by weight. The animals are kept in their cages for at least 5 days prior to the start of the study to allow for acclimatisation to the laboratory conditions. During this period, the animals should be placed in the nose-only restraint tubes on the exposure system for up to 6 hours/day for at least 4 days and exposed to filtered air at similar conditions that will be used during the study.

The fibre under test is administered by inhalation of an atmosphere of aerosolised fibres. The method of aerosolization is dependent on the physical chemical properties of the fibre. The method chosen should not contaminate the fibres and it should minimise possible alteration of the fibre surface or the production of non-fibrous dust (through excessive grinding or abrasion of the fibres).

1.4.2 Test conditions

1.4.2.1 Experimental animals

The Wistar rat supplied specific pathogen free (SPF), virus antigen-free (VAF+) and maintained under optimum hygienic conditions (OHC) is preferred. Upon receipt and at selected intervals throughout the study, a sentinel group of rats should be analysed for bacteriological and viral contamination. Another strain of laboratory rat may be used providing it is validated in comparison to the Wistar.

The animals should be maintained under barrier conditions throughout the study. The rats should be acclimated to the animal room used for housing during the test for at least 5 days after clinical health examination. The rats should be approximately 8 - 10 weeks old with a weight range of ± 20 % of the mean at the start of the acclimation period. The exact age and weight should be recorded as part of the study data.

1.4.2.2 Number and sex

At least 10 rats (males are preferred) /group/sacrifice time point should be used. For the air control group, 10 animals/group/sacrifice time point should be used.

1.4.2.3 Animal room and housing during non-exposure periods

Animals should be housed in either stainless steel wire or polycarbonate cages. During the exposure period, cages for housing when the animals are not being exposed should be arranged in such a way that possible effects due to cross contamination of fibres from one group to another are minimised. If 2 or more test fibres are included in the study, each fibre group should be sufficiently isolated to minimise cross contamination between groups.

1.4.2.4 Choice of Exposure Concentrations (Dose Levels)

Animals should be exposed to three concentrations of the test substance. The fibre exposure aerosol should be prepared to be largely rat respirable and have:

- A mean aspect ratio of at least 3:1,
-

- The exposure concentrations stated below refer to the number of fibres with geometric mean length greater than 20 μm. The geometric mean diameter of those fibres longer than 20 μm should be as close to 0.8 μm as possible, for fibres with a density $\rho \cong 2.4$, if technically feasible. For fibres with densities different from this the corresponding GMD should be determined. (Note: The GMD varies as the square root of the density for a constant median aerodynamic diameter).
-
- The gravimetric mean concentration of those fibres, which are 0.8 μm or less, should not exceed 40 mg/m^3 in the exposure aerosol, if technically feasible.
- As an upper limit, the gravimetric concentration of all particles (fibrous and non-fibrous) in the test atmosphere should not exceed 60 mg/m^3, if technically feasible.

The aerosol concentrations to which the animals are exposed should be:

1. 15 fibres/cm^3 longer than 20 μm in length with a GMD as close as possible to 0.8 μm.
2. 50 fibres/cm^3 longer than 20 μm in length with a GMD as close as possible to 0.8 μm.
3. 150 fibres/cm^3 longer than 20 μm in length with a GMD as close as possible to 0.8 μm.

The need for multiple doses will be re-examined following the accumulation of sufficient data.

A control group of animals exposed to filtered air only is to be included. The treatment of the control animals should be performed under the same conditions as for the animals receiving the test fibres.

It should be noted that diameters could be smaller than 0.8 μm if justified by the dimensions of the bulk fibre.

1.4.2.5 Characterisation of the Test Article used for aerosol generation

The chemical composition of the fibre material supplied for testing to at least within 0.5 % and the density of these fibres should be provided.

1.4.2.6 Duration and Frequency of Exposure

Each group of animals should be exposed for 6 hours/day, 5 days/week for 13 consecutive weeks.

1.4.2.7 Observation period

Sub-groups of animals should be sacrificed at:

- The termination of exposure (Following study day 90)
- After 45 days of non-exposure (Following study day 135)
- After 90 days of non-exposure (Following study day 180)
- Other time points if appropriate

1.4.2.8 Laboratory Validation Fibre

Prior to starting studies on test fibres, laboratories with no previous experience in testing fibres should analyse a validation fibre by performing either a similar exposure regime as described herein or a 5-day inhalation biopersistence study. All laboratories should analyse the validation fibre according to either protocol if no similar test has been carried out in the last 5 years. Fibres such as E-glass, MMVF 21 or MMVF 10a are recommended as the validation material. Optionally other fibres could be used as validation material if sufficient data are available.

1.4.3 Procedure

1.4.3.1 Test Article Preparation

In order to achieve the requirements stated in section 1.4.2.4, the bulk fibre used for aerosol generation should be prepared or pre-selected by size to be respirable in the rodent. As a general guideline, for fibres of density $\rho \cong 2.4$, a geometric mean diameter as close to 0.8 µm as possible and a geometric mean length of approximately 15 µm will facilitate achieving those requirements.

If a pre-selection process is used or the bulk fibres used are produced by a non-commercial production method, a validation must be included in the study report which shows that the fibre has similar chemical and surface characteristics as compared to that produced commercially.

1.4.3.2 Fibre Aerosol Generation system

The fibre aerosol generation system must be capable of producing the required aerosol concentration of fibres as described above continuously for a period of 6 hours/day without contaminating the fibres, altering the fibre surface or producing non-fibrous dust (through grinding or abrasion of the fibres). A suitable charge neutraliser (e.g. Ni63) should be placed immediately following the aerosol generator to assure that the fibres are discharged to Boltzman equilibrium.

The system should be able to produce the required aerosol exposure with a mean uniformity of plus or minus 15 % based upon gravimetric measurements of aerosol concentration.

1.4.3.3 Inhalation Exposure System

It is recommended that the flow-past nose-only exposure system (refs. (3), (4)) be used. Other nose-only exposure systems may be used if they are validated with respect to equivalent or greater fibre deposition in the lung of fibres > 20 µm in length as compared with the flow-past system.

The testing facility must provide documentation showing that the uniformity of fibre concentrations at the top, middle and bottom level of the exposure system is within ± 15 %.

1.4.3.4 General observations

1.4.3.4.1 MORTALITY

All animals should be observed for mortality/moribundity before the start and after the completion of each exposure and at least once on non-administration days

1.4.3.4.2 CLINICAL SIGNS

Each animal should have a detailed clinical observation for signs of toxicity, including time of onset, intensity and duration:

- at least once during acclimatisation phase
- at least twice weekly during the exposure phase.
- at least once weekly during the recovery phase.

1.4.3.4.3 BODY WEIGHT

All animals should be weighed at least

- once at the beginning of acclimatisation period,
- on the day of first exposure, prior to the start of exposure,
- once weekly thereafter through week 13,
- once every second week thereafter.

1.4.3.4.4 GROSS NECROPSY

As a control of animal health, all animals in the study should be subjected to a full, detailed gross necropsy, which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. All animals should be anaesthetised and killed by exsanguination. All gross necropsy findings should be recorded.

1.4.3.4.5 ORGAN SAMPLING

At each examination time point, a total of 10 animals/group should be sacrificed with allocation as follows:

1) 5 animals/group/time point allocated to:

a) Fibres lung burden analysis (Right lobes) and

b) Histopathological examination and proliferative response following administration of BRDU (Left lobe).

2) 5 animals/group/time point - Inflammatory response (Bronchial alveolar lavage, BAL).

All animals should be autopsied as follows except those assigned to the BAL group in point 2 above.

- The lungs and the lower half of the trachea should be removed and weighed.
- The right main stem bronchus leading to the right lobes should then be ligated and these lobes removed, weighed and immediately deep-frozen.

- The remaining lung (left lobe) should then be inflated with an appropriate fixative by instillation under a pressure of 30 cm H_20 and fixed by immersion for a minimum of 2 hours in the fixative .

In animals assigned to the BAL group in point 2 above:
The lungs with the lower two thirds of the trachea should be sampled, observed for macroscopic findings (naked eye), and immediately submitted to broncho-alveolar lavage as described below (1.4.3.4.5.3).

1.4.3.4.5.1 Lung Burden

In order to permit the association of the histopathological, proliferative and inflammatory response indices with lung dose:

The right lobes should be stored at -20°C. These lobes should be freeze dried or critical point dried as quickly as possible thereafter and within two weeks of sacrifice to minimise possible fibre dissolution. Other appropriate methods can also be used. These lobes are allocated for lung digestion and subsequent fibre analysis.

Following drying of the right lobes, the dry lung weight should be determined. The dry tissue should then be digested using an appropriate method. For mineral fibres the preferred method is low temperature plasma ashing.

All tissue digestion methods should be validated prior to use. Using a standard addition procedure and a minimum of 3 previously unexposed rat lungs/dose group, doses of 0.05, 0.1 and 0.5 mg of a similar well characterised fibre should be injected and the lungs digested. The mean fibre number should be within ± 25 % of what was injected and the size distribution recovered should not be statistically different from that injected.

1.4.3.4.5.2 Histopathological examination and Proliferative response

Histopathological examination:

The ventral half of the left pulmonary lobe, divided longitudinally (if indicated, additional parts of the lungs may also be processed), the trachea and relevant gross lesions in any organ/tissue from all animals should be embedded in paraffin, cut at a nominal thickness of 4 micrometers /slide and stained with hematoxylin and eosin. Other stains can be used if appropriate and multiple slides/sections are recommended. An additional set of slides from the lung should be stained with a collagen specific stain.

All slides from all animals should be examined by a pathologist. All abnormalities should be described and included in the report. Evaluations of lung tissue should include assessment of collagen deposition at the broncho-alveolar junction, pulmonary fibrosis, interstitial fibrosis, peribronchiolar lesions and pleural changes. The severity of fibrotic response and the area of the lung showing fibrosis should be quantified.

Proliferative Response:

In the right median and accessory lobes, tissue of the terminal bronchioles, mesothelial cells and lung parenchyma cells should be examined for cell proliferation using the sensitive S-phase response method. Proliferating cells should be labelled by 5-bromo-2'-deoxyuridine (BrdU) which should be administered to the animals. The preferred method is to remove the animals from exposure two days prior to sacrifice and to apply the BrdU using a mini-pump. The lung section slides should be prepared according to histological routine procedures and stained immuno-histochemically. The evaluation of the slides should be done by analysing airway cells and cells of the proximal regions of the pulmonary parenchyma.

1.4.3.4.5.3 Inflammatory response

The lungs with the lower two thirds of the trachea should be sampled, observed for macroscopic findings (naked eye), and immediately submitted to broncho-alveolar lavage with a modified Hank's solution (or saline). Following tracheal cannulation, the airways should be lavaged at least 2 times each with 4 ml of Hank's solution.

The pooled lavage fluid for each rat should be collected in a centrifugation tube on ice. This fluid should be centrifuged at approximately 300 g for 10 minutes. Two ml of the supernatant should be collected and analysed for enzymatic activity of lactate dehydrogenase (LDH), and for determination of total protein. Other markers can also be used if appropriate.

The pellet should be collected and aliquots taken for analysis of total and differential cell count.

1.4.4 Study Monitoring

1.4.4.1 Exposure system monitoring

- Airflow rate (monitored continuously and recorded at least once per day)
- Oxygen concentration (at least once per day). The oxygen concentration in the vicinity of animal's nose should be maintained at a level of at least 19.5 %. If the flow-past nose-only exposure system is used and the airflow supplied to each animal is at least 1 l/min, then it is not necessary to measure the oxygen concentration.
- Temperature & humidity of the air supply (at least once per day). The temperature should be maintained at 22 ± 2 °C. To achieve the fibre aerosol exposures, it is recognised that the supply air to the generator can be dry and as such no lower limit is placed upon humidity. Review of the air control groups from a series of chronic studies has shown that there is no adverse effect from such low humidity.

1.4.4.2 Exposure atmosphere monitoring and analysis

All sampling for measurement of the aerosol exposure concentration and size distribution should be performed near where the animal's nose would be in the exposure system.

For the air control group, the sampling duration should be as long as possible (approximately 3 to 5 hours) in order to permit the assessment of the absence of contamination.

Sufficient monitoring of the exposure atmosphere should be performed during the pre-study phase in order to assure that the required fibre aerosol concentrations and uniformity are achieved during the study. If a flow past exposure system is not used, sampling should be performed using methodology designed to minimise anisokinetic sampling errors.

The analyses specified below should be considered as the minimum analyses that should be performed. If anomalies in the results occur, additional filters should be analysed, if available.

1.4.4.2.1 GRAVIMETRIC (mg/m^3)

If gravimetric concentration is used for monitoring of the aerosol fibre number, aerosol mass monitoring should be performed daily for a duration representative of the daily concentration. Daily sampling should be performed for at least 2 hours per day with each individual sample of at least one-hour duration. The gravimetric concentration should be determined from each filter sampled and expressed in mg/m^3.

1.4.4.2.2 FIBRE AND PARTICULATE NUMBER (fibres/cm^3) AND BIVARIATE SIZE DISTRIBUTION (µm) BY SCANNING ELECTRON MICROSCOPY (SEM)

These should be sampled at least once per day. These samples should be taken in timely coincidence with the gravimetric sampling with sample duration dependent upon fibre type (usually less than 30 minutes). One filter per day during the first week and two samples/week thereafter should be analysed for fibre and particulate number, with the remaining filters used if anomalies are found. Bivariate analysis of diameter and length should be determined at least twice weekly with the additional filters used if anomalies are found. Fibre concentration should be expressed as total number of fibres/cm^3 and the number of fibres/cm^3 with length > 20 µm, 5-20 µm, < 5 µm and WHO fibres and the number of particles/cm^3.

1.4.4.2.3 CHEMICAL ANALYSIS

One filter sample should be taken for possible analysis.

1.4.4.3 Counting and Sizing Rules (for aerosol and lung fibres)

The general guidelines provided by the WHO/EURO (ref. (1)) are recommended with the following additional procedures for mineral fibres.

1.4.4.3.1 LENGTH AND DIAMETER

Sizing of length and diameters should be performed using a SEM at a magnification of at least 2000. All objects, which are seen at this magnification, are to be counted. Fibres crossing the boundary of the field of view should be counted as follows. Fibres with only one end in the field are weighted as half of a fibre and fibres with neither of their ends in the field are not measured. Diameters of fibres which are seen at 2000 magnification should be measured at 'full screen' magnification (usually up to a magnification of

10,000). No lower or upper limit is to be imposed on either length or diameter. For bulk fibres with mean diameters below a few tenths of a micrometer, an initial magnification of at least 5000 should be used. The length and diameter are to be recorded individually for each fibre measured so that the bivariate distribution can be determined. When sizing, an object is to be accepted as a fibre if the ratio of length to diameter was at least 3:1. All other objects are considered particles. There should be no truncation in the measurements. If fibre measurements are made using SEM photomicrographs or video prints where the magnification of the photo or print is at least twice that of the SEM screen, then an initial SEM magnification of at least 1000 is acceptable, providing fibres of 0.1 μm diameter can be resolved. When using photomicrographs or video prints, higher magnifications should be used for diameter measurement than for length measurement in order to ensure good precision.

1.4.4.3.2 STOPPING RULES

Enough fields of view are to be counted for evaluation so that at least a total of 0.15 mm^2 of the filter surface (for 25 mm diameter) is examined. Once this condition is fulfilled:

1. Fibres: The evaluation of fibres should be stopped when 400 WHO (L > 5 μm, D < 3 μm) fibres are counted/measured for each sample (lung, filter, etc.) analysed by SEM, or a total of 1000 fibres and non-fibrous particles were recorded, or 1 mm^2 of the filter surface was examined, even if a total of 400 countable WHO fibres was not reached. Otherwise, the procedural variability and counting errors could result in a false estimate of the measure. The total number of fibres per filter should be determined by normalising the surface area counted to the total surface area of the filter.
2. Particles: The recording of particles can be stopped when a total of 30 particles are counted. If the size distribution of particles is measured, care should be taken in the lung samples to confirm by EDAX which particles are of the same composition as the fibres.

Other strategies for measurement (such as size selective analysis using a minimum of 100 fibres per category with at least 3 length categories) can be used providing that the method has been validated to produce similar results statistically in comparison to the above method.

2 DATA

2.1 ANIMAL DATA

Individual data should be provided. Additionally all data should be summarised in tabular form showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons and the time of any death or humane kill, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the number of animals showing lesions, the type of lesions and the percentage of animals displaying each type of lesion. In addition, the body weights and lung weights should be provided.

2.2 FIBRE CHEMISTRY

The chemical composition of the fibre material provided for testing to within at least 0.5 % and the density of the fibre tested should be presented.

2.3 EXPOSURE AEROSOL

The following parameters should be reported for the exposure aerosol:

2.3.1 Fibres

Number of fibres evaluated microscopically; Mean and standard deviation gravimetric concentration (mg/m^3); Mean Total Fibres/cm^3; Mean WHO Fibres/cm^3; Mean number of fibres > 20 µm in length/cm^3; Mean number of fibres < 5 µm, 5-20 µm and of WHO size per cm^3; Diameter range (µm); Length range (µm); Mean and standard deviation Arithmetic Diameter (µm); Mean and standard deviation Arithmetic Length (µm); Geometric mean diameter (µm) and Geometric standard deviation; Geometric mean length (µm) and Geometric standard deviation.

2.3.2 Particles

Number of Particles evaluated microscopically; Mean Number of Particles/cm^3.

2.4 LUNG BURDEN

At each sacrifice time point and for each animal, the following parameters should be reported for each group:

2.4.1 Fibres

Number of fibres evaluated microscopically; Mean Total Fibres/lung; Mean WHO Fibres/lung;
Mean number of fibres > 20 µm in length/lung; Mean number of fibres < 5 µm, 5-20 µm and of WHO size per lung, Diameter range (µm); Length range (µm); Mean and standard deviation Arithmetic Diameter (µm); Mean and standard deviation Arithmetic Length (µm); Geometric mean diameter (µm) and Geometric standard deviation; Geometric mean length (µm) and Geometric standard deviation.

2.4.2 Particles

Number of Particles evaluated microscopically; Mean Number of Particles/lung.

2.4.3 Summary Tables

In addition a summary table showing the following data for each time point should be provided:

Fibre type; study group; sacrifice time point (days); Mean total number particles/lung Mean number and percent remaining of WHO fibres/lung; Mean number and percent remaining of fibres/lung in the following length categories: < 5 μm; 5-20 μm and > 20μm.

2.5 HISTOPATHOLOGY

Data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

In addition, the amount of collagen deposition at the broncho-alveolar junctions should be presented as a grade from 0-5 (see Annex). The chronic pulmonary changes and fibrosis should also be evaluated according to a grading system such as that presented by McConnell et al. (ref. (5)). The severity of fibrotic response and the area of the lung showing fibrosis should be presented.

2.6 PROLIFERATIVE RESPONSE

Summary tables by group and individual data showing the number of proliferating cells /area for each sacrifice time point.

2.7 INFLAMMATORY RESPONSE

Summary tables by group and individual data showing the levels of lactate dehydrogenase (LDH) , and total protein, total cell number and differential cell counts.

2.8 DATA ANALYSIS

Results should be evaluated by an appropriate statistical method. Any recognised statistical method may be used.

3 REPORTING

3.1 TEST REPORT

The final report should include but not be limited to:

- The identification of test material, either by name or code number.
- The composition and other appropriate characteristics of the test fibre.

- A description of the test rats, including strain, source, number, allocation, sex, body weight range, age, method of identification, housing, diet etc.
- A description of the exposure concentrations, exposure regimen and duration of the treatment periods.
- A description of all methods.
- A description of all results.
- All statistical results as described in Section on Data Analysis and Method above.
- Summary tables as described above.
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- Figures of body weights.
- Summary tables of antemortem clinical signs, mortality data, body weights and pulmonary lobes weights.
- Individual tables of body weights, lung burden data, pulmonary lobes weights and necropsy findings. Tables on histopathiological, proliferative response and inflammatory response data.

. Discussion of the results.

. Interpretation of the results.

4 REFERENCES

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ANNEX

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EPS scoring system for Collagen Deposition at the Bronchiolar Alveolar Junction (1)

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Severity scores are assigned as follows:

Grade 0 is characterized as no remarkable changes (refers to a normal lung).

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Grade 2 is characterized by slight, fairly easily detected, few, small foci of collagen deposition. Lesions of this severity represented the lowest level of grade 4 in the Wagner-scoring system.

Grade 3 is characterized by moderate, easily detected foci of collagen deposition in considerably enlarged areas at the bronchiolar-alveolar junction. Lesions of this severity also represented grade 4 in the Wagner-scoring system.

Grade 4 is characterized by marked, obvious or extensive foci of collagen deposition extending from the bronchiolar-alveolar junction into the interstitium of more peripheral parts of the lung parenchyma. Lesions of this severity also represented grade 4 in the Wagner-scoring system.

Grade 5 is characterized by severe, widespread collagen deposition with consolidation at the bronchiolar-alveolar junction, sometimes with interlobular linking. Lesions of this severity represented grade 4 to 5 in the Wagner-scoring system.

Wagner-scoring system (2)

The histologic scoring system according to the criteria given by Wagner et al. (2), is also used to describe collagen deposition. In this scoring system a pulmonary change, grade 1.0 is considered normal, grades 2.0 and 3.0 are characterized by cellular changes (alveolar macrophages, microgranulomas, bronchiolization), and grades 4.0 to 8.0 represent the former lesions plus increasing distribution and degrees of fibrosis. The degree of fibrosis may range from minimal to moderate, but is consistent with grade 4.0 as long as no interlobular linking of the aforementioned lesions occurs. Grades 2.0 and 3.0 are considered potentially reversible while grades 4.0 to 8.0 are thought to be non-reversible.

REFERENCES

(1) Jorg Chevalier, Experimental Pathology Services AG, CH-4132 Muttenz (Basel), Switzerland. Personal communication.

(2) Wagner, J.C., et al., The effects of the inhalation of asbestos in rats, British journal of Cancer, 29, 252-269 (1974).

Annexes

Annex 1

ECB/TM/21(97)

MEETING ON FIBRES COUNTING AND SIZING
Ispra, 24 September 1997

TESTING METHODS FOR MMMF

GUIDANCE NOTES ON PREPARATION OF SAMPLES, SETTING OF THE MICROSCOPE AND COUNTING/SIZING PROCEDURES

PREPARATION OF THE SAMPLES

The following indications are useful to convert the lung digestate to liquid suspension, for other sample types as bulk fibre, aerosol or suspensions, only the relevant parts apply.

1- The whole lung should be digested.

2- The entire digestate should be suspended in liquid.

3- Liquid for suspension: Absolute methanol is recommended as the solvent (e.g. 20 ml)

4- Homogeneisation method: A suitable ultrasonic water bath that does not alter the fibres should be used. As an example a Bransonic® ultrasonic cleaner 1210, with a maximum power of 143 W at 47 KHz has shown to be useful for this purpose.
Use a glass bottle of 25 ml, containing 20 ml of absolute methanol. Sonicate for 1 minute and look at the suspension through the bottle in front of a light. If clusters remain, sonicate again during 1 minute. Repeat until no evident clusters remain.

5- Aliquot: 0.5 – 2.0 ml of suspension should be used for each sample to ensure proper loading of the filter.

6- Loading of filters: should not exceed 10 fibres / 10,000 μm^2 of SEM screen field.

7- Filter media: 25 mm diameter polycarbonate filters of 0.2 μm pore size should be used (for example Nucleopore®). They should be pre-gold-coated to a thikness of 40 nm.

8- Filtration:

A) - Filter Holder: Should be either in glass or steel (for example Millipore®)
- A back-up filter not gold coated but otherwise the same as the main filter should be placed behind the gold coated filter.
- Both filters should be placed on a support pad to ensure uniformity.
- The filters support pad should be placed on an etched stainless steel back-up support.

B) Prior to filtration, the filter already placed in the holder should be covered with 5 ml of absolute methanol.

C) The selected aliquot should be pipetted onto the methanol layer in the filter holder.

D) After the aliquot is in the holder, moderate vaccuum is turned on to slowly aspirate the methanol until there is no obvious liquid remaining but not to dryness.

E) Additional absolute methanol should be used to rinse and clean the walls of the filter holder.

9- The sample should be examined by SEM to ensure uniformity and to control the loading and the general quality of the sample. If the loading uniformity or the quality is not appropriate, the filters should be discarded and a new aliquot taken.

10- Post coating of filters with gold is not recommended.

MICROSCOPE SETTING

11- The microscope used should be a Scanning Electron Microscope with Energy Dispersive Spectroscopy attachement.

A) Adjustement: The minimum visible diameter, using a real sample matrix, at a magnification of 2,000x should be less than 0.2 μm.

B) Callibration: Should be performed at least once a week using a certified callibration grid.. The SEM should be adjusted so that callibration lines are within ± 2 %.

COUNTING/SIZING PROCEDURE

12- All fibres seen at magnification 2,000x should be taken into account. To measure the diameter, the magnification should be increased to "full screen" (ca. 10,000x). Additional thin fibres seen at this higher magnification, if any, should not be counted.

13- Where particles are counted, EDS should be used to verify their identity.

14- The use of videoprints is not recommended because they can lead to truncation of results by disregading long fibres that go beyond the photo field and because an enormous amount of prints would be required to achieve equivalent resolution of diameters.

Annex 2

LIST OF MEETINGS

1996

- July 8-9. Ispra. Ad-hoc Experts' Meeting on Testing Methods for MMMF.
- September 24. Ispra. 22nd Meeting of the National Co-ordinators for Annex V to Dir. 67/548/EEC.
- November 12-13. Ispra. 2nd Ad-hoc Experts' Meeting on Testing Methods for MMMF.

1997

- 21 January. Ispra. Satellite Meeting on MMMF.
- 6-7 March. Ispra. Meeting on MMMF
- 15 April. Ispra. 23rd Meeting of the National Co-ordinators for Annex V to Dir. 67/548/EEC.
- 2-3 June. Geneva. Meeting on Correlations between Short Term Tests and Chronic Tests for MMMF
- 21-23 July. Ispra. Ad-hoc Experts' Meeting on Testing Methods for MMMF.
- 24 September. Ispra. Meeting on Fibres Counting and Sizing.

1998

- 25 February. Ispra. 24th Meeting of the National Co-ordinators for Testing Methods of Annex V to Dir. 67/548.
- 11-12 May. Ispra. Meeting on Validation/Callibration of Testing Methods for MMMF.
- 8 September. Ispra. 25th National Co-ordinators Meeting.
- 30 November. Ispra. Meeting on calibration of the 90 days subchronic inhalation test for MMMF

Annex 3

LIST OF PARTICIPANTS IN THE MEETINGS

W. Allescher
L.K. Andersen
J. Ahtiainen
I. Angelopoulou
A. Auletta
G. Balodis
C. Beausoleil
P. Bechmann
D. Bernstein
H. Biedermann
R. Binetti
S. Bintein
P. Brochard
R. Brown
P. Brunko
C. Burley
K. Cameron
J. Cheron
G. Corcelle
J. Costa David
P. Di Prospero
K. Donaldson
J. Fentem
K. Furtmuller
T. Garlanda
J. González García
N. Gregg
D. Hanton
J. Hart
A. Hesbert-Laudet
M. Hof
M.C. Huet
D. James
O. Kamstrup
W. Karcher
C. Kazuhiko
M. Kolossa
H. Komulainen
P. Kristensen
K. Grodzki
T. Lakhanisky
M. Leynen
C. Lambre
A. Lange
C. Lasne
A. Lundgren
A. Marconi
R. McEneany
M. Meldrum
C. Morscheidt
H. Muhle
T. Neustadt
H. Nover
G. Oberdorster
E. de la Peña
G. Pessina
I. Pratt
M. Reisner-Oberlehner
E.M. Reiss
J.M. Riego Sintes
B. Rihn
H. Roelfzema
F. Schorsch
P. Sebastien
L. Seedorf
E. Sundquist
U. Teichert
R.S. Tregunno
H. Tyle
E. Valcarce de Angulo
P. Wardenbach
R. Warner
H. Witzani
A. Young

Many other experts provided comments during the several written commenting rounds, their names are not included in this list for the sake of simplicity.

This document was prepared by

David M. Bernstein, Ph.D.
Consultant in Toxicology

40, ch. De la Petite-Boissière
CH-1208 Geneva
Switzerland

Juan M. Riego Sintes, Ph.D. *
Testing Methods Area Co-ordinator
European Chemicals Bureau

European Commission Joint Research Centre
Institute for Health and Consumer Protection
Unit: Toxicology and Chemical Substances.
T.P. 280
21020-Ispra (VA), Italy

* To whom correspondence should be addressed.