

# Registration of Allergen preparations

NORDIC GUIDELINES

Prepared by the Nordic Council on Medicines in cooperation with the Drug Regulatory Authorities in Denmark, Finland, Iceland, Norway, Sweden

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## Introduction

Preparations of allergens intended for use in the diagnosis and treatment of allergy are subject to national jurisdictions and regulations applying to pharmaceutical specialities.

The documentation submitted for registration of allergen preparations as pharmaceutical specialities must satisfy the general directives formulated by the drug control authorities. These directives are in the following referred to as the general guidelines (Drug Applications, Nordic guidelines).

The present supplementary instructions comprise guidelines for the specific documentation required for the registration of allergen preparations as pharmaceutical specialities. Several of the sections in these instructions include explanatory notes and comments of non-mandatory nature. They primarily comprise more detailed recommendations regarding e.g. the methodology to be applied in the production and control of allergen preparations.

Testing required for routine control of each batch is specified under Quality specifications (3.7). Everything else forms part of the basic documentation.

The documentation submitted shall be well-arranged and complete, with appropriate reference sections ordered in the same sequence as in these instructions. If parts of the documentation are the same for several registrations applied for, it is permissible to refer to a specific masterfile.

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## 1. General information

The name and composition of the preparation, dosage form, kind and size(s) of packages, manufacturer, shelf life and storage precautions shall be stated.

A short description should be given stating the source and manner of processing of the allergens in the preparation and the principle for standardization of its composition and potency.

A short summary of the chemical and immunochemical characteristics should also be presented in this section. Particulars of indications, directions for use, contraindications, adverse reactions, warnings and precautions and dosage are to be stated.

## 2. Standards of manufacture

Allergen preparations shall be manufactured under conditions which comply with the Code of Good Manufacturing Practice (GMP) as established by the WHO or in the Pharmaceutical Inspection Convention (PIC), an agreement entered into by a number of European States. If the manufacturer operates in a country with which there is an agreement on mutual recognition of inspection of drug manufacturers, the drug control authorities can ask for an inspection report when required. Any other manufacturer whose production standards are not known to the authorities must prove that his operations comply with the Codes of Good Manufacturing Practice referred to above.

### 3. Chemical and pharmaceutical documentation

#### 3.1 Synopsis of chemical and pharmaceutical documentation

See Drug Applications, Nordic guidelines.

#### 3.2 Complete composition

The name (scientific name, e.g. genus and species as well as any common name), and type (e.g. pelt, dander, saliva) of the allergenic source material(s) and the biological potency shall be stated. The names, grades and quantities of all additives shall also be given. The composition of reconstituting or diluting liquid shall also be stated.

#### 3.3 Starting materials

##### 3.3.1 Source materials

The allergenic source material shall be described in as much detail as possible. The description shall include particulars concerning collection, pretreatment and storage. The name and address of the supplier shall be stated. Specifications and control methods for the source material(s), applied by the supplier, shall be included as well as the specifications and control methods used by the manufacturer of the allergen preparation. The specifications shall ensure that the qualitative and quantitative composition of the material is as uniform as possible from one delivery to another. They shall encompass requirements and control methods relating to identity and purity. The source materials should be purchased from firms which are specialists in dealing with allergenic materials. The source materials should be stored under such conditions that changes are kept to a minimum.

##### *Pollens*

The manner of collection of pollens shall be stated. Tests for content of foreign pollens, spores, extraneous plant material from the same species and non-related contamination shall be included. The collection of pollens shall be supervised by a person with botanical expertise.

*The pollen content from other species should be limited to 1 % of mixed pollens and 0.5 % of a single pollen as determined by a microscopic particle count; detectable spores should not exceed 1 %; other contamination, including*

*Collection of hair or dander must take place using methods which provide a good epithelial harvest without injuring the skin of the animal. Methods employing the grinding of whole skins/pelts must not be used. When killed animals are used the epidermals must be collected within a few hours or the animals must be stored in conditions ensuring that postmortem decomposition processes do not affect the epithelium.*

##### *Hymenoptera venoms*

The method of collection of isolated venom proteins from the venom sacs of Hymenoptera species shall be described. The collection of Hymenoptera venoms shall be supervised by a person with entomological expertise.

*The venom protein shall be isolated from the venom sacs by appropriate methods ensuring that the raw material is of a proper quality.*

##### 3.3.2 Intermediate and bulk products

When production is started from a raw extract or other intermediate or bulk products, the identity and quality specifications of this starting material can be stated in a certificate obtained from the supplier. The certificate shall define the source material, the procedure used for collection, extraction etc as specified above and shall specify the identity and quantity of the allergenic components. The documentation shall moreover specify the tests and the frequency of testing performed by the manufacturer.

If full information on the preparation of an intermediate or bulk product is not available to the manufacturer, the supplier of the product may provide detailed information in a master file submitted directly to the national authorities.

### 3.3.3 Other starting materials

The manufacturer's quality specifications shall be submitted in the manner stated in the general guidelines (Drug Applications, Nordic guidelines).

*Ingredients such as buffers, antimicrobial agents and stabilizers, as well as other materials used in the manufacturing process such as defatting agents and ingredients of the extraction solution, are presumed to be of the same quality as starting materials used in the manufacture of other drugs. The same applies to the ingredients of reconstituting liquids included in the packages. Ingredients commonly recognized as immunogenic or allergenically active in humans should not be used in the production of allergen preparations.*

### 3.4 Packaging material

Data on packaging material shall be supplied as directed in the general guidelines (Drug Applications, Nordic guidelines).

### 3.5 Method of manufacture

Documentation on factories involved in the manufacture of the allergen preparation and reconstituting liquid shall be submitted in accordance with the general guidelines.

The entire production process shall be described, step by step, with a diagram (flow-chart) indicating the principles of the process, accompanied by an explanatory text. The different stages of the manufacturing process, such as grinding, extraction, filtration, clarification, dialysis, concentration, fractionation, sterilization, filling into final containers, lyophilization etc, must be clearly defined. The description shall state the stage at which aseptic precautions are introduced. Intermediate or bulk products in the process shall be identified and the in-process controls performed at these or other stages of manufacture reported.

*The production process should comprise a purifying stage to remove nonimmunogenic contaminations below a relative molecular mass of normally about 5,000. If, however, it can be shown in individual cases that special lowmolecular weight components are relevant for the specific activity of the product, this molecular weight cut-off may be changed. The principle of the purification and fractionation methods shall be defined, and it should be clearly apparent at which step in the process special biochemical techniques are used.*

### 3.6 Basic documentation

The documentation shall contain relevant information regarding the development of the product and the chemical and immunochemical characterization of the preparation.

As far as possible, data should be provided which demonstrate that the allergen preparation is representative, i.e. contains the relevant allergens of the allergenic starting material.

Detailed information shall be given regarding the methods applied, where necessary with relevant literature references.

#### 3.6.1 Standard preparations

Standardization of an allergen preparation requires the establishment of a welldefined, characterized and stable reference preparation.

##### 3.6.1.1 In House Reference Preparation (IHR)

A representative batch of the allergen extract shall be established as the IHR for the product in question. The IHR shall represent a prototype for all further batches of the allergen extract (intermediate or bulk product), thus the qualitative composition of regular production batches should match the IHR. The IHR shall be characterized using available relevant methods (see 3.6.3) and its specific biological activity shall be established (see 3.6.2). The presence of all relevant allergens in the IHR shall have been demonstrated in comparative studies involving several batches of raw extract.

The stability of the IHR shall be ensured by proper sampling and storing.

*The IHR should be stored at low temperature (-20 °C), preferably freeze-dried in ampoules. A prediction of its thermal stability may be obtained by accelerated degradation tests, determining relative degradation rates of samples stored at elevated temperatures compared with samples stored continuously at -20 °C. The stability studies shall include assays for total allergenic activity as well as tests capable of detecting and estimating changes in the content of the relevant allergens.*

#### 3.6.1.2 International Standard Preparation (IS)

Where an IS is established, documentation on comparative studies with the IHR is required, using qualitative and quantitative methods.

*The IS should be used according to recommendations formulated by the Allergen Standardization Subcommittee of the International Union of Immunological Societies (IUIS) Provided that the IS and the IHR give parallel lines in the assay of total allergenic activity (RAST -inhibition assay) the IS shall be utilized to calibrate the potency of the IHR in International Units.*

#### 3.6.2 Biological standardization

This item defines the principles for biological calibration of the In House Reference Preparation (IHR). The biological activity of the IHR shall be determined by skin prick testing in humans, using the reference method described separately in the Annex. The unit of activity is defined on the basis of this biological assay (see below). Where alternative methodology is used, documentation must be provided showing the equivalence with the NLN method.

*The following definition is used for the unit of activity: the activity of an allergen extracts 10,000 Biological Units (BU) per ml when the extract provokes a specific skin reaction in the median sensitive patient with a wheal of the same size as a wheal provoked by a positive reference solution consisting of histamine 54.3 mmol/l (for example histamine dihydrochloride 10 mg/ml) when both solutions are administered using the same technique (prick testing) on at least 20 individuals who are clinically allergic and cutaneously reactive to the allergen concerned.*

For subsequent batch testing (see Quality specifications, 3.7) a suitable in vitro assay (e.g. RAST inhibition) may be used, with the IHR as reference.

#### 3.6.3 Chemical/immunochemical characterization

Data shall be provided regarding protein and where relevant carbohydrate content. Characterization of protein, including distribution of antigens and allergens, should be determined by means of relevant biochemical and immunochemical techniques. Where relevant, investigations performed with the IHR (3.6.1.1) may be referred to.

*In the course of development of the product its chemical properties in terms of protein content shall be investigated by several methods to ensure that full information is obtained about its characteristics. Some of the following methods should be applied: crossed immunoelectrophoresis (CIE), isoelectric focusing, electrophoresis in polyacrylamide gel, determination of the distribution of molecular weight by SDS (sodium dodecyl sulphate) gel electrophoresis and quantitative determination of total protein.*

*Information regarding the allergenic specificity of the proteins in the extract may be obtained from experiments involving combinations of electrophoretic methods and immunoblotting techniques or crossed radioimmuno-electrophoresis (CRIE). Sensitivity spectra (allergogram) derived from such experiments on individual patients' sera should be included in the basic documentation, thus identifying the major, intermediate and minor allergens. As far as possible the individual allergens should be identified using internationally accepted nomenclature (cf. MIS recommendations regarding allergen nomenclature: Bull. World Health Org. 64(5), 767-770 (1986)), or the correspondence with allergens described in the scientific literature should be given,' including literature references.*

### 3.7 Quality specifications

Quality specifications shall include all requirements and test methods applied in quality control. The manufacturer shall specify the frequency of testing and the intermediate or finished product to which the tests are applied. Specifications shall normally encompass identity, potency and purity as well as pharmaceutical and technical properties of the preparation and packages.

Properties of the IHR used in batch control can be described in section 3.6, since identification of the antigen/allergen component(s) and determination of potency shall be based on the basic documentation.

The specifications shall define the allergen components in the preparation, as well as the content of the important allergen components relative to the content of the same components of the IHR. The requirement applied to total allergenic activity in every batch shall be stated.

Data obtained from at least two separate batches shall demonstrate the reproducible composition of the product.

The choice of methods used should be discussed. The control methods shall be described separately in sufficient detail for them to be carried out in an independent laboratory.

Specification of the quality requirements applied to non-active ingredients and packages shall follow the general guidelines for other products as outlined in the Drug Applications, Nordic guidelines.

The requirements set forth in the quality specifications shall apply throughout the shelf life of the preparation(s).

#### 3.7.1 Assay of total allergenic activity

Standardization of the total allergenic activity of individual batches of an allergen extract should be undertaken, preferably by RAST inhibition or by direct RAST. An account must be given of the producer's internal standardization of the method and the reagents, as well as of the criteria applying to the reagent serum (e.g. patient inclusion criteria, pool size and specificity). The requirements for assessing parallelism between standard and test samples in the assay shall be given.

Each individual batch shall be assayed against the biologically standardized In House Reference Preparation (IHR). The potency of the batch can then be declared on the basis of the BU system (see 3.6.2).

Where an IS has been established and the IHR has a corresponding qualitative composition, the total allergenic activity of each individual batch shall also be expressed in International Units (cf. 3.6.1.2).

When a product consists of one or a few well-characterized allergenic components, standardization can be undertaken by assay of the individual component(s) by means of alternative relevant techniques, such as single radial immunodiffusion, quantitative immunoelectrophoresis or other quantitative techniques. In such a situation, too, the standard(s) used shall be calibrated according to the BU system (see 3.6.2).

The estimated potency derived from the assay of total allergenic activity should be not less than 50 % and not more than 200 % of the stated potency. A validation of the assay method shall show that it gives a relative standard error of the estimated potency which is less than or equal to 20 %. All relevant components of variation should be included in the standard error.

#### 3.7.2 Determination of the antigenic components

The constant composition of the product can be ascertained by relevant separation methods and compared with that of the In House Reference Preparation. Thus, the antigenic/allergenic components shall be identified and estimated, e.g. by CIE/CRIE or by combinations of different electrophoretic methods and immunoblotting techniques. The preparation and specificity of the antibodies/anti sera used in these assays shall be described.

#### 3.7.3 Determination of protein composition

The reproducible protein composition of the product shall be shown by suitable electrophoretic methods. Sensitive techniques like polyacrylamide gel electrophoresis, isoelectric focussing in suitable gels or SDS gel electrophoresis in reducing or non-reducing media can be applied. The staining techniques used and the design of the analyses should allow semiquantitative estimation of the individual protein components.

The total protein content shall be determined by a micro-Kjeldahl technique or by another relevant method.

#### 3.7.4 Other tests

The specifications shall also describe other tests and the requirements applied to define the properties and the purity of the product. Allergen preparations shall be sterile. Other tests may include water content and tests for abnormal toxicity (not relevant in the case of preparations intended for skin prick testing only).

To describe the methods used, reference may be made to those described in the European Pharmacopoeia (Ph Eur, Ed II) or other widely accepted pharmacopoeias.

#### 3.8 Modified allergen preparations

In some cases, relevant identity, potency or purity tests cannot be applied to the finished product of an allergen preparation. For chemically modified, precipitated or adsorbed allergen preparations the allergen components can no longer be solubilized and studied in their native form. In such situations quality specifications shall be defined for the intermediate product obtained prior to modification of the allergen extract.

Standardization of modified allergen preparations may be based upon protein determination before and after modification and determination of total allergenic activity (e.g. by RAST inhibition) before modification.

For all modified preparations, limits for the remaining free total allergenic activity shall be stated in the quality specifications of the finished preparation (cf 3.7).

#### 3.9 Stability

The stability of the allergen preparation shall be documented in accordance with the general guidelines . Hence, the stability report shall show that the preparation retains acceptable activity and, where possible, allergen composition throughout the shelf life claimed.

The stability of the preparation when subjected to conditions similar to those of the usage period shall be described. This also applies to reconstituted products and dilutions of allergen preparations. Stability data on reconstitution and dilution liquids shall also be submitted.

Storage and transport conditions shall be stated.

#### 3.10 Labelling

Information about labelling of the product shall be given in accordance with the general guidelines. Detailed information is given in Labelling of Proprietary Medicinal Products (NLN Publication No 13).

The biological activity of the preparation shall be declared in Biological Units (BU, see 3.6.2). For modified allergen preparations, the declared activity shall express biological activity before modification. Information regarding free allergenic activity (cf. 3.8) shall be given in the package insert.

*Where an international standard can be applied, the activity of the preparation shall be expressed in IU, e.g. in the package insert, together with a statement of the method used.*

*For pure, well-defined allergen preparations the concentration may be given in mass/volume units.*

### 3.11 Samples

Sample packages and samples of ingredients are to be submitted concurrently with the application, as provided in the general guidelines.

On request necessary reagents for control of the preparation shall also be submitted.

## 4. Toxicological and pharmacological documentation

### 4.1 Synopsis of toxicological and pharmacological documentation

See Drug Applications, Nordic guidelines.

### 4.2 Toxicology

The safety of allergen preparations from a toxicological point of view must in principle be evaluated along the same lines as those applicable to other pharmaceutical specialities intended for injection. Documentation on toxicology can include studies concerning acute toxicity (diagnostic and therapeutic preparations) and prolonged toxicity (therapeutic preparations only).

*The feasibility and relevance of conventional toxicity testing may vary and the results may be questionable, since possible immunological side effects may be difficult to differentiate from general toxic reactions. The need for careful attention by clinicians in the clinical trials of the preparation must therefore be stressed (section 5).*

*However, the following general guidelines are suggested:*

**Acute toxicity testing** (diagnostic and therapeutic preparations): Two animal species should be used (e.g. mouse and guinea pig or rat); calculated on a per kg body weight basis, the dose injected should be 300-3,000 times the highest dose intended for clinical use; the animals should be observed for 1-2 weeks and their weight gains or losses recorded; the animals should then be subjected to macroscopic postmortem anatomical-pathological examinations.

**Subacute toxicity testing** (therapeutic preparations only): Two animal species should be used; 1/20-1/110 of the dose level used in the acute study should be given daily over at least 3-4 weeks, or alternatively the injection may be performed once a week and the administration period extended to at least 12 weeks. After sacrifice the animals should be examined as in the acute test. Where required a histopathological examination should be performed.

*Toxicology testing is not necessary for diagnostic preparations intended for skin prick testing.*

Tests performed on each production batch shall be described in section 3.7.

## 5. Clinical documentation

### 5.1 Synopsis of human pharmacological and clinical documentation.

See Drug Applications, Nordic guidelines.

Presentation of the documentation should follow the general guidelines

The sensitivity and specificity (in relation to disease) of allergen preparations intended solely for diagnostic skin prick test purposes are documented by skin prick testing according to the Annex.

Aqueous preparations for conjunctival, nasal or bronchial challenge tests must meet the same quality requirements as other diagnostic allergen preparations and be standardized using the same procedures.

For therapeutic preparations more extensive reports on clinical trials are required.

## 5.2 General

The main purpose of the clinical trials is to prove the therapeutic value of the product and to enable an effective and safe dosage schedule to be recommended.

The documentation should provide information about

- the aims of the investigation
- diagnostic criteria and/or indications for treatment
- the number of patients
- selection of patients; criteria for inclusion or exclusion (duration and severity of disease, age and sex distribution, previous therapy, other concurrent treatment)
- randomization
- study design (open, controlled, double-blind, etc)
- dosage (e.g. based on dose finding studies), mode of administration
- duration of treatment
- instructions given to the patient and information about how the course of treatment was recorded
- how the effect was documented (skin prick test, challenge tests, symptom scores and immunochemical methods)
- how adverse reactions were investigated e.g. what laboratory tests were performed
- results and statistical analysis, stating the methods used and reasons for their use.

The number of clinical trials necessary to establish therapeutic efficacy will depend on the type of the extract, the nature of the disease, the reliability of the methods of evaluation used etc. Controlled trials in different centres are usually required. In certain cases with a known natural history, open studies with a restricted number of patients may be acceptable if a clinical effect of the treatment is documented using objective methods such as provocation tests.

*Well-defined diagnostic criteria for the selection of the study population should be applied. Inclusion and exclusion criteria and rules on cessation of therapy must be defined in the protocol. The effect should be observed by objective methods of measurement, but laboratory parameters alone are not sufficient documentation of clinical efficacy. Appropriate and well-defined effects must always be included as criteria of clinical efficacy. The sample size must be sufficient for statistical analysis of the results.*

The duration of the studies may vary depending on the purpose of the trials and the nature of the allergen preparation. It is important to take into account any spontaneous and seasonal variation of the disease, as well as changes in patient compliance which are likely to occur.

Evaluation of results should usually include at least one analysis of all the patients allocated to treatment and control groups, including all withdrawals ('Intention to treat analysis'). If such an analysis is not performed, the reasons for this should be given. Concomitant therapy during a clinical trial must be discussed.

Patients of both sexes in different age groups should be investigated. The number of drop-outs must be clearly stated and the reasons for their withdrawal from the study given.

### *Combined allergen preparations*

In addition to the requirements mentioned above, the following clinical documentation should be given for combination preparations:

Clinical trials must be reported showing the therapeutic value of the combination. It must also be documented or motivated that the fixed combination has advantages over administration of one of the active ingredients separately for the majority of patients for whom the preparation is intended.

### 5.3 Adverse reactions

Special attention has to be paid to the recording of adverse reactions.

The nature and frequency of adverse reactions have to be investigated as a part of the clinical trials.

The overall clinical documentation must generally include data on large and representative groups of patients, irrespective of the indications, and these patients should be fully monitored for clinical and biochemical adverse reactions. The exact requirements will necessarily vary with the nature of the allergen preparation and the disorder, the known adverse effects of related compounds and findings from animal experiments and human immunology. Details of the method of ascertaining adverse reactions are to be given. The results should be subjected to statistical analysis and the method used and the reason for its use stated. Naturally, this fully monitored group will, as a rule, only comprise part of the total clinical experience. Data on individual patients who have received the preparation for longer periods should be presented if available.

### 5.4 Dosage

Recommended dosage based on the dose finding studies and clinical trials shall be presented. If the dosage for children is stated, it must be documented by special studies. Attention should also be paid to major individual variations from patient to patient and also to in-patient variation.

### 5.5 Overdosage

Data available on cases of overdosage shall be given. Recommendations on how to treat patients who have received an overdose should also be given, based on general knowledge of the substance and experience from patients who for some reason have been given an overdose. Unexpected immunological reactions caused by overdosage should be mentioned in this section if they are known.

## 6. Maintenance of registration

Significant changes in the manufacturing process must be approved by the drug control authorities. Applications for such approval should be accompanied by test results and relevant documentation showing the total allergenic activity and antigen composition of the product manufactured according to the amended process in comparison with the registered product.

In addition, changes in suppliers of allergenic raw material or in the quality of raw materials, changes in packaging materials, changes in the composition or quality specifications of the intermediate or bulk product or finished preparation, and major changes in control methods shall be reported.

New research data of biochemical, immunological, toxicological or clinical relevance to an evaluation of the preparation must be submitted. Follow-up studies on clinical properties and on stability should be reported regularly to the drug control authorities.

## **Annex**

### Quantitative skin prick test procedure for biological standardization of allergen preparations

#### **Introduction**

The present skin testing procedure is the reference method for the biological calibration of In House Reference Preparations. The method is not intended for routine testing of allergen extracts. The use of this procedure is the basis for the definition of the unit of activity: 'The activity of an allergen extract is 10,000 Biological Units (BU) per ml when the extract provokes a specific skin reaction .in the median sensitive patient with a wheal of the same size as a wheal provoked by a positive reference solution consisting of histamine 54.3 mmol/l (for example histamine dihydrochloride 10 mg/ml), when both solutions are administered using the same technique (prick testing) on at least 20 individuals who are clinically allergic and cutaneously reactive to the allergen concerned.'

The results of biological standardization by means of skin testing are dependent on the sensitivity of the allergic individuals. Therefore the criteria for choice of patients for testing must be strictly followed. The histamine reaction is primarily used to control the technique of the operator with regard to reproducibility on the same patient and to compensate for differences in skin reactions caused by differences in technique between operators. Biological standardization allows comparisons of relative potencies between allergen preparations prepared from different source materials. Using preparations labelled with Biological Units for diagnosis, valuable information is obtained regarding the relative degree of sensitivity to different allergens. However, without further documentation, the Biological Unit does not reflect the clinical efficacy of therapeutic allergen preparations, and is no general basis for dosage in immunotherapy.

The biologically standardized In House Reference Preparation (IHR) is part of the basic documentation of the product. The batch-to-batch consistency of potency, expressed in Biological Units/ml, which is one of the factors contributing to the safety of the allergen preparation, is ensured by in vitro methods, e.g. RAST inhibition.

#### **Definitions and abbreviations**

A - Wheal reaction. This is recorded as the area of the wheal (mm<sup>2</sup>).

Ah - Geometric mean of the wheal reactions provoked by the 10 mg/ml histaminemine dihydrochloride reference solution in the individual patient.

C - Concentration (mg dry weight /ml) or relative concentration of allergen preparation.

Ch - Histamine equivalent concentration: calculated (relative) concentration of the allergen preparation theoretically provoking a wheal reaction of size A<sub>hi</sub> in the individual patient.

H1 - Histamine dihydrochloride solution 1.00 mg/ml

H10 - Histamine dihydrochloride solution 10.0 mg/ml

#### **Patients**

Preferably at least 20 patients should be used for the biological standardization procedure. The patients must have given informed consent to participate. Patients should not recently have been exposed to allergen, e.g. biological standardization using grass pollen sensitive patients should be performed outside the grass pollen season.

#### *Inclusion criteria*

1. The patients should live in an area where allergy caused by the allergen concerned is a relevant problem.  
Test subjects should be chosen consecutively or from a register at a university hospital or a unit with corresponding competence. Patients should not be excluded due to low or high sensitivity to the allergen, when they otherwise fulfil the inclusion criteria.
2. A positive case history with inhalant allergy related to exposure to the allergen to be tested.
3. A positive prick test (mean wheal diameter  $\geq$  3 mm) when tested with a standardized extract prepared from the allergen source in question and/or a positive test for specific IgE.
4. A mean wheal diameter  $\geq$  4 mm obtained in a prick test with histamine dihydrochloride 1 mg/ml.
5. Age: preferably 16-50 years (median age of the group of patients should be between 20 and 30 years); both sexes.

### *Exclusion criteria*

1. Immunotherapy in the past 5 years with an allergen preparation known to interfere with the allergens to be tested.
2. Use of drugs which may interfere with the skin reaction.

In addition to drugs normally used in the treatment of allergy, i.e. antihistaminic drugs, other drugs may influence the results of skin testing. Therefore, patients participating in the biological standardization of an allergen extract must interrupt the use of such drugs for a period of time to eliminate the influence on the skin reactivity. The length of this period of time should be based on available documentation concerning any interference with the skin reactivity. It will depend on i.a. the plasma half-life of the drug, also considering any inter-individual variations of drug metabolism.

3. Pregnancy, dermatographism, atopic dermatitis (locally at the test site), urticaria.

### **Materials**

1. Allergen preparation: freeze-dried In House Reference Preparation.
2. Solutions of histamine dihydrochloride 1.0 mg/ml and 10.0 mg/ml in water for injections. Requirement for the potency of the histamine solutions: 0.90 - 1.10 mg/ml and 9.0 - 11.0 mg/ml of histamine dihydrochloride, respectively, when assayed according to the monograph on Histamine Phosphate Injection of USP XXI, p. 491 (the calculation must be adapted for assay of histamine dihydrochloride).
3. Albumin diluent: a solution containing 0.9% (w/v) sodium chloride, 0.03% (w/v) human serum albumin and 0.4% (w/v) phenol.
4. Equipment for dilution of the allergen preparation: vials with caps, disposable syringes, needles.
5. Syringes and needles or a dropper plastic cap for application of drops of allergen on the skin.
6. Prick test lancet; a lancet with a 1 mm long point and shoulders preventing the needle from penetrating the skin further than 1 mm.
7. Transparent adhesive tape.
8. Ballpoint pen or fine filter tip pen.
9. Test result sheet for proper recording of results.

### *Preparation of dilutions for testing*

Reconstitute the freeze-dried extract with a volume of albumin diluent giving a suitable concentration of allergen preparation. This is the stock solution.

Prepare four parallel series of five ten-fold dilutions from the stock solution. Starting with the highest concentration, number the vials from 6 (the stock solution) to 1.

Precautions: Use a new syringe and needle for each ten-fold dilution. Do not flush or reaspirate air or diluent into the needle or syringe when the more concentrated solution has been added to the diluent vial. Keep the dilutions at +4°C. Prepare new dilutions every day.

Use the albumin diluent as negative control in the prick test procedure.

### **Skin prick test procedure**

#### *Principle of the assay*

In each patient three ten-fold concentrations of allergen are tested together with the histamine reference solution, in quadruplicate. The three concentrations of a of the patient (see "Preliminary test"). The dose-response relationship of the allergen is estimated by regression line analysis using the geometric mean of the four wheal areas obtained with each concentration, in a double-logarithmic system. The concentration of allergen estimated to provoke a response with the same wheal area as the histamine reaction (geometric mean of the four wheal responses caused by HIO) is then calculated from the regression line formula. The median value based on all patients tested represents the concentration of the allergen preparation corresponding to 10,000 BU/ml.

#### *Practical performance of skin prick testing*

Place a drop of the solution on the skin. Press the needle (the lancet) at a 90° angle into the superficial layer of the skin through the drop. Apply the pressure for one second. Apply the same pressure every time. Then wipe off the drop with a soft tissue.

#### *Design of the assay*

##### Preliminary test

Apply in duplicate concentrations nos. 1, 2 and 3 together with solution HI (in duplicate) on the volar surface of the forearm. For the individual patient select as the lowest dose in the final test the concentration giving a wheal of approximately the same size as that of HI.

### Final test

Apply in quadruplicate on the back of each patient the concentration of allergen selected in the pre-test and the next two higher concentrations, together with solution H10 in quadruplicate. Include, for control purposes, HI and a negative control solution (albumin diluent), also in quadruplicate. The allocation of test sites is demonstrated in figure 1. These should be placed at least 4 cm apart.

### *Recording of skin reactions*

15 minutes after application of each solution onto the skin, encircle the contour of the wheal using the skin marking pen: draw the line on the red skin surrounding the wheal, taking care not to cross the wheal or cover any part of it, and to ensure that no part of the red skin surrounding the wheal appears inside the encircled part. Gently press the transparent tape against the wheal and transfer the contour to the record sheet. Measure the area of the wheal with a digitizer by following the contour lines.

### *Calculation of results*

Replace any wheal area = 0 (e.g. undetected wheal reactions of the allergen preparation) with wheal area = I mm<sup>2</sup>, and calculate for each patient the geometric mean of the four wheals provoked by each of the three allergen concentrations and by H10 (Ali). For each patient, using the method of least squares, perform linear regression analysis and compute the constants a and b to describe the relationship:

$$\log (A)=a+b \log (C)$$

Insert the value for Ali into the equation and compute the corresponding C (=Ch)

The principle of the calculation is illustrated in figure 2; an example with data from I patient is added (table 1).

Repeat the procedure for all patients.

The median Ch for all patients represents the concentration corresponding to 10,000 BU/ml. For an odd number of patients (n) the median Ch is the Ch for patient no. (n+1)/2 of the rank; for an even number of patients (n) the median Ch is the mean of the Ch for patient no. (n/2) and the Ch for patient no. (n/2)+1.

The data from each patient, taken from the test result sheet, may be summarized in a form as demonstrated in table 1.

### Calculation of 95% confidence limits

The patient number of the rank representing the lower and upper 95 % confidence limit respectively can be taken from table 2.

(The 95 % confidence interval for the median Ch from 20 patients is given by the Ch for patient number 6 and the Ch for patient number 15).

### *Requirements/criteria for assay validity*

The operator performing skin prick testing must have established a reproducible technique. With histamine dihydrochloride 1 mg/ml wheal areas between 20 and 50 mm<sup>2</sup> should be obtained, with a coefficient of variation  $\leq 40\%$  (20 consecutive tests in the same patient).

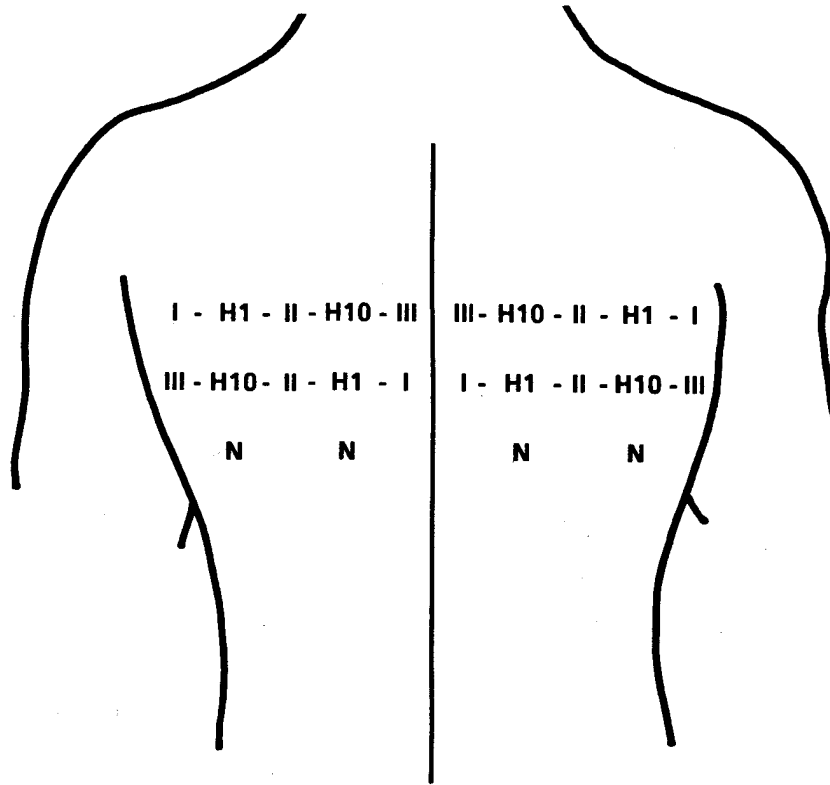
Accept only results from individual patients fulfilling the following criteria:

- Geometric mean of the wheal reactions provoked by either of the two highest concentrations of the allergen preparation  $\sim 7$  mm<sup>2</sup>.
- Geometric mean of the wheal reactions provoked by H10  $\sim 7$  mm<sup>2</sup>.
- Geometric mean of the wheal reactions provoked by the negative control  $< 7$  mm<sup>2</sup>.
- Slope of regression line  $\sim 0.1$
- Correlation coefficient  $\sim 0.85$

The pooled standard deviation of the four log wheal areas obtained with all concentrations of the allergen preparation in all patients from the centre should be less than 0.4.

# Attachment I

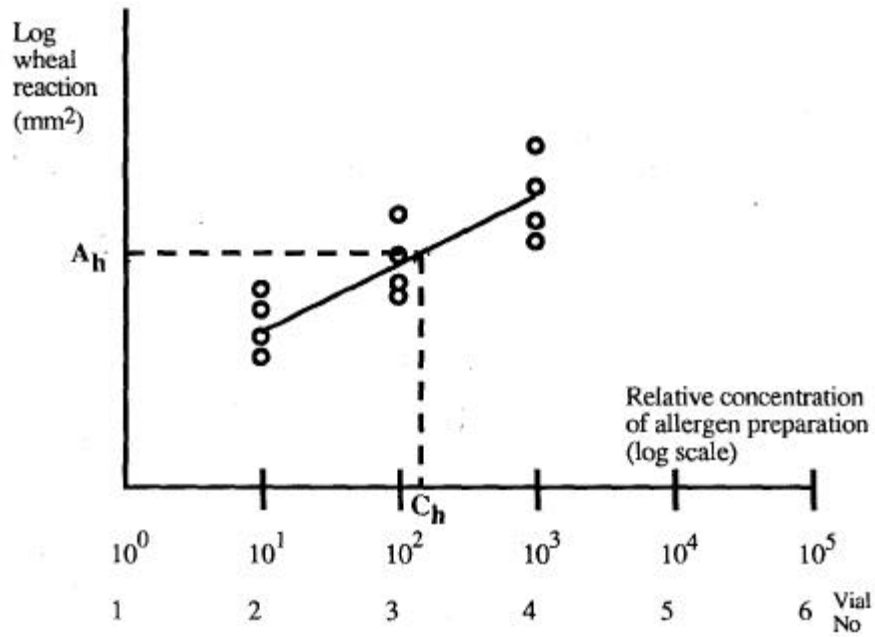
Figure 1: Allocation of test sites



<u>Site</u>		<u>Relative conc.</u>
I	Allergen extract solution	I
II	"	10
III	"	100
HI	Histamine diHCl 1 mg/ml	-
H10	Histamine diHCl 10 mg/ml	-
N	Neg. control (albumin diluent)	-

Attachment 2

Figure 2: Principle for calculation of the histamine (10) wheal equivalent (relative) concentration of allergen preparation in one test subject



Regression line:  $\text{Log} (A) = a + b \text{Log} (C)$

- Ah Geometric mean of the wheal reactions elicited by the 10 mg/ml histamine dihydrochloride reference solution
- Ch The corresponding (relative) concentration of allergen preparation (eliciting a wheal reaction = Ah)

**Attachment 3**

Table I (example):

Skin prick test results: Patient no. I

Wheal area (mm<sup>2</sup>)

ALLERGEN PREPARATION					REFERENCE/CONTROL		
VIAL NO.		2	3	4	H10	H1	N
REL.*		10	100'	1000,			
CONC		μG/ML					
R E P L I C A T E	1	27	32	75	35	15	4
	2	20	35	49	49	17	3
	3	13	4-7	95	28	13	2
	4	17	27	46	30	12	3
GM		19	35	63	Ah=35	14	

H10: Histamine dihydrochloride solution 10 mg/ml

H1: Histamine dihydrochloride solution 1 mg/ml

N : Negative control (albumin diluent)

GM: Geometric mean

\* Relative concentration of the stock solution (vial no. 6) = 10<sup>5</sup>; relative concentration in vial no 1 = 10<sup>0</sup>.

Allergen regression line:  $\text{Log (A)} = 1.02 + 0.26 \text{ Log (C)}$   
Slope = 0.26

Correlation Coefficient = 0.99

Log Ch = computed after insertion of Ah into the equation of the allergen regression line

Ch = 103

Attachment 4

Table 2

Confidence limits (> 95%)

n	Rank of lower limit	Rank of upper limit
10	2	9
11	2	10
12	3	10
13	3	11
14	3	12
15	4	12
16	4	13
17	5	13
18	5	14
19	5	15
20	6	15
21	6	16
22	6	17
23	7	17
24	7	18
25	8	18
26	8	19
27	8	20
28	9	20
29	9	21
30	10	21
31	10	22
32	10	23
33	11	23
34	11	24
35	12	24
36	12	25
37	13	25
38	13	26
39	13	27
40	14	27
41	14	28
42	15	28
43	15	29
44	16	29
45	16	30
46	16	31
47	17	31
48	17	32
49	18	32
50	18	33