

## STANDARDIZATION OF HOUSE DUST MITE ALLERGEN (A13 *D. PTERONYSSINUS*)

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### ABSTRACT

*The introduction of new in vitro methods, recommended by the European pharmacopoeia (1), enrich the characterization and evaluation of the allergens. The standardization of house dust mite allergen (A13 Dermatophagoides pteronyssinus) is based on different methods for determination of its structure and biological activity. Five batches of allergen were standardized by determination of protein nitrogen, by isoelectric focusing and by assessment of major allergen Der p1 by ELISA. The biological activity of the A13 was evaluated by skin prick test (SPT), IgE-binding potential after competitive inhibition and its activation capabilities for in vitro basophil degranulation. These batches, produced from 2002 - 2006, were with identical protein distribution according their isoelectrical points. Der p1 and protein nitrogen quantities varied between 37 – 87 ng/ml and 39 - 34 mg %. The studied lots showed similar results in the IgE-binding properties in comparison with the reference at 50 % inhibition level and in flow cytometry test for allergen-specific basophil degranulation. All the batches were with similar biological activity and induced skin-allergic reactions, comparable with the in-house reference preparation and histamine hydrochloride standard 1 mg/ml. The data from these new, in vitro methods improve the results from the accepted standardization procedure and will be performed as routine in the evaluation of biological activity of the house dust mite and others allergens.*

**Keywords:** standardization, house dust mite allergen, skin prick test, protein content, isoelectric focusing, ELISA, competitive inhibition, flow cytometry basophil test

**Abbreviations:** SPT - skin prick test; IHRP - in-house reference preparation

### Introduction

Consistent high-quality allergen extracts are essential for accurate diagnosis and effective treatment of allergic disease (5). The goal of allergen standardization is to produce well-characterized extracts of known biologic potency and composition with minimal variations from lot to lot. This process requires the selection of an appropriate reference extract and methods to compare the test extract with the reference. Until recently the main methods for standardization of allergen extracts were determination of protein content, isoelectric focusing and skin allergometry (8). The implementation of new methods will promote more detail characterization of allergens and more precise evaluation of their biological activity.

The aim of this study is to introduce the complementary *in vitro* methods for standardization of house dust mite allergen (A13 *Dermatophagoides pteronyssinus*) and to compare the results of five batches with the in-house reference preparation (IHRP) and with accepted standardization procedures.

### Materials and Methods

#### House dust mite allergens

Five lots of house dust mite allergen A13 *D. pteronyssinus*: 01 2002-OP, 01 2003-OP, 01 2004-OP, 01 2005-OP, and 01 2006-OP, manufactured in Laboratory of allergy at National center of infectious and parasitic diseases, were studied.

#### Determination of protein content

The method for estimation the quantity of protein nitrogen after decomposition with a sulphuric acid is used (3).

The amount of protein nitrogen in the allergen extracts should be greater than 1 mg %.

#### Isoelectric focusing

This method achieves distribution of the extract's proteins according their isoelectric points in agar's plate with definite pH gradient after the direct current impact by use of the Multiphor II electrophoresis system (LKB).

The Ampholine PAG plate pH 3.5-9.5 (GE healthcare) is used. 10 µl of the allergen samples and the standards (Isoelectric focusing calibration kit, pH range 3-10, Pharmacia, LKB) are distributed by their isoelectric points in time and conditions, recommended from the PAG plate's producer. After the end of the process, the plate is processed with fixing, washing and staining solutions and the proteins distribution by isoelectric points and the whole protein profile of the extract is read.

### Determination of extract's total allergen activity by *in vivo* method of skin allergometry

Each new produced A13 *D. pteronyssinus* allergen lot is applied by SPT in 3 dilutions on sensitized patients. The in-house referent preparation, the histamine hydrochloride standard 1 mg/ml and the negative control (50 % glycerinated Coca solution) are applied simultaneously (6, 7). The allergy skin reaction (the size of the weal and the flare) is read after 20 minutes. The median diameter of each test and control solutions is calculated on the basis of the weal's size from each tested patient. IHRP should provoke skin reaction (weal) with median diameter equal to this of the histamine standard. The dilution of the tested allergen lot, which cause skin reaction equal or  $\pm$  1 mm of the reaction of the IHRP and of the histamine standard is specified to obtain the total allergen activity equal to 1000 BU/ml.

### Determination of Der p 1 by ELISA

The ELISA kit (Indoor Biotechnologies) is used to define the quantity of Der p1 according to the product protocol (4).

Polystyrene microtiter plates (NUNC) are coated with anti Der p1 mAb 5H8. After washing, 1% BSA is added and plate is incubated for 30 min. The 0.1 ml of doubling dilutions of the Der p1 standard from 250 to 0.5ng/ml and 0.1ml of diluted allergen are placed in wells. After incubation and washing diluted biotinylated anti Group 1 allergens mAb 4C1 is adding. The plate is incubated and washed and diluted Streptavidin - Peroxidase is added. The assay is developing by adding 0.1ml o-phenyldiamin. The plate is read at the 492 nm optical density and quantity of Der p1 (ng/ml) in tested allergen lots is estimated toward standard control curve.

### Assessment of IgE-binding potential after competitive inhibition

The ImmunoCAP (Phadia, AB) is used to investigate IgE-binding properties in allergen extracts after competitive inhibition according method's protocol (14).

Preliminary determinate serial dilutions of A13 *D. pteronyssinus* allergen lots (5-folded and/or 10-folded) are prepared in PBS. 250  $\mu$ l of patients' sera or pooled sera are placed to each tube and equal volumes of diluted allergens are added. For zero percentage and for 100 percentages of inhibition, 250  $\mu$ l of PBS and 250  $\mu$ l of no diluted allergen extract are added at the first and at the last tubes. The incubation is done using gently shaker for 2 hours at room temperature. The quantity of specific IgE is estimated by ImmunoCAP 100 (Phadia, AB). The percentage of inhibition is evaluated according the formula:

$$\left( \frac{0\% \text{ inhibition value} - x\% \text{ inhibition value}}{0\% \text{ inhibition value} - 100\% \text{ inhibition value}} \right) \times 100$$

### Basophile's activation test

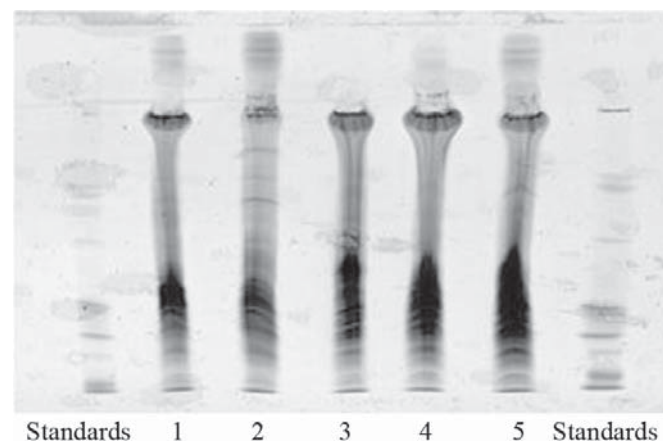
Flow cytometry kit BD Fast Immune test (BD) for quantitative determination of allergen-specific basophils degranulation is used. Sample preparations were performed according the kit's instructions. Peripheral blood was collected in a vacutainer tube with sodium heparin (BD).

The stimulation of the blood samples and activation with A13 *D. pteronyssinus* allergen is done simultaneously (1). For negative and positive controls, the stimulation buffer only or diluted N-Formil-Met-Leu-Phe (fMLP) are used. The samples are analyzed by flow cytometer FACSCalibur (BD) with a 488 nm argon laser. At least 1000 basophiles were acquired for each sample. The basophils are defined by two analytical gates and are SSC<sup>low</sup> CD123+ HLA-DR-. The degranulated basophils are with phenotype CD63+CD123+HLA-DR- and the percentage of these cells is measured by FL1 histogram.

## Results and Discussion

House dust mites are the most important source of indoor allergens (5, 10, 11). Allergen extract of *D. pteronyssinus* is unique instrument for precise diagnosis of persisting allergic rhinitis, bronchial asthma and atopic dermatitis and useful tool for specific immunotherapy (12, 13). The allergen batches should be with constant antigen structure and similar allergen activity i.e. they should be standardized. The proper standardization requires availability of reference preparation for house dust mite allergen. The Laboratory of allergy, National center of infectious and parasitic diseases, take a part in the elaboration of the international reference from *D. pteronyssinus*, which was approved in 1984 as the First international standard WHO/IUIS *D. pteronyssinus* NIBSC 82/518 by the WHO Expert committee for biologic standardization (9).

However, this international reference has established only for research and for *in vitro* purposes. Therefore the European pharmacopoeia recommends the use of in-house reference preparation for the routine standardization of commercial allergen extracts (2). IHRP is well characterized by the protein content determination, a protein profile, identification of relevant allergens and determination of total allergenic activity *in vivo* and *in vitro* (8). This reference is used for assessment of new produced lots by accepted procedures and is a criterion for introduction of new *in vitro* laboratory methods, which give us an opportunity to expand the allergens standardization procedure



**Fig. 1.** Isoelectric focusing of house dust mite allergen lots **1** - Lot № 01 2002-OP; **2** - Lot № 01 2003-OP; **3** - Lot № 01 2004-OP; **4** - Lot № 01 2005-OP; **5** - Lot № 01 2006-OP

Determination of protein nitrogen immediately after extraction is the first step of the standardization of house dust mite allergen. As it is shown on the **Table 1**, the amount of protein nitrogen in all studied batches is greater than 1 mg % - between 38.68 – 74.19 mg % (average 49.01 mg %). The quantity of the protein nitrogen indicates that the extraction procedure is passed properly and represents presence of proteins, which have allergenic potential.

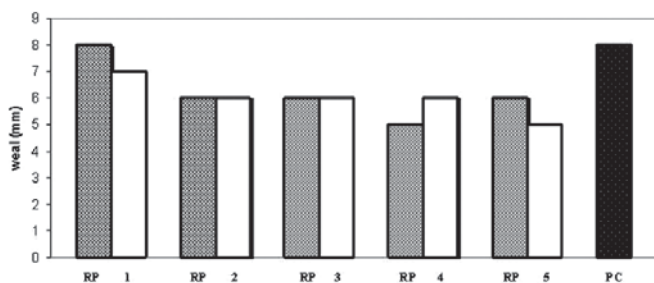
**TABLE 1**

The protein nitrogen content and the quantity of major mite allergen Der p1 in five batches of house dust mite allergen (A13 *D. pteronyssinus*).

Lot N	Protein nitrogen amount (mg %)	Der p1 contents (ng/ml)
01 2002-OP	74.19	50.21
01 2003-OP	38.68	71.51
01 2004-OP	53.20	129.71
01 2005-OP	39.68	54.71
01 2006-OP	39.63	42.80

Assessment of different lots by isoelectric focusing should demonstrate the similarity between the numbers and the location of the protein bands, which confirm the extract's identity. The protein profiles of the studied lots of the house dust mite allergen are presented on **Fig. 1**. The proteins in all allergen lots are concentrated mainly in the area with pH 3-5. There is clear resemblance between the protein bands of studied allergen extracts, which demonstrate that all lots have the similar protein profile.

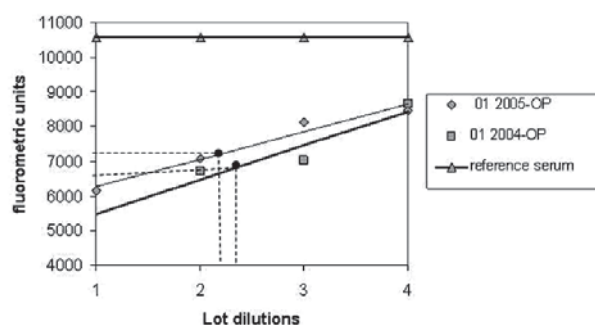
The summarised data of batches standardisation as regards their total allergen activity *in vivo* are presented on **Fig. 2**. The median diameter size of the weal is in the range 5 – 7 mm for all studied new lots and is equal or  $\pm 1$  mm of the reaction of the corresponding IHRP. The comparison of the results from SPT demonstrates the constancy of the *in vivo* allergen activity of mite allergen lots. This provides a guarantee for stabile efficacy in the diagnosis and the treatment of house dust mite allergy.



**Fig. 2.** Determination of extract's total allergen activity by skin allergometry  
**RP** - in-house reference preparation; **1** - Lot № 01 2002-OP; **2** - Lot № 01 2003-OP; **3** - Lot № 01 2004-OP; **4** - Lot № 01 2005-OP; **5** - Lot № 01 2006-OP; **PC** - histamine hydrochloride 1 mg/ml

The amount of Der p1, one of the major *D. pteronyssinus* allergens, is determined by ELISA with monoclonal antibodies.

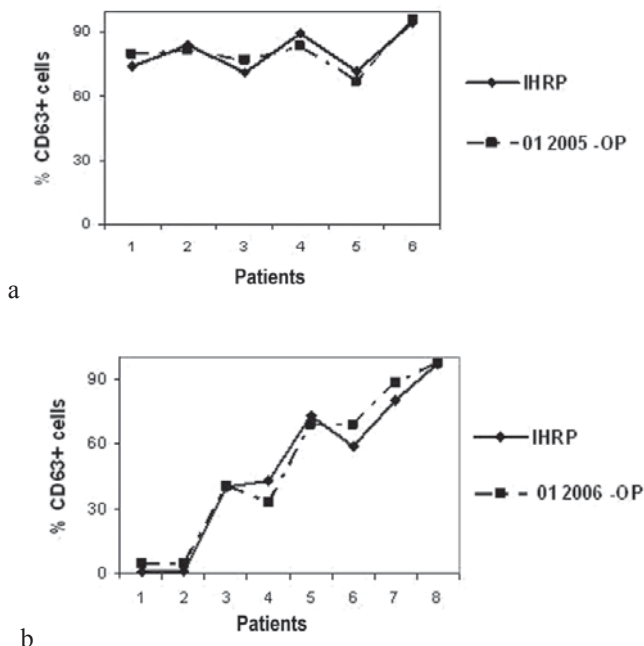
Thus the house dust mite extracts are characterized as regards the levels of this important protein, bearer of the main allergenic activity, which is an indirect marker for the total biological activity of the preparation. The quantities of Der p1 in the studied lots of house dust mite allergens are presented in the Table 1. The results demonstrate that the major allergen's amounts are in range between 42 – 130 ng/ml (average 70 ng/ml). ELISA is one of the suitable methods, recommended by the European pharmacopoeia for assessment of individual allergens. The obtained values of Der p1, determined by this method, should be in the range from 50 to 200 % of the state amounts (2). Our results show that Der p1 quantity in all analyzed A13 *D. pteronyssinus* batches is in the calculated range 35 – 140 ng/ml and the lot-to-lot variations are minimal.



**Fig. 3.** IgE-binding properties of different lots from A13 *D. pteronyssinus* assessed by ImmunoCAP inhibition.

The IgE-binding properties of allergen extracts were investigated by ImmunoCAP inhibition. The results from IHRP and from five new lots of house dust mite allergen are compared. The reference extract was used to establish level for 100% inhibition and the linear regression on the data between 10 and 90% inhibition were performed. The degree of similarity between the lots was represented by the slope of the lines - 793.3 for lot № 01 2004-OP and 983 for lot № 01 2005-OP (**Fig. 3**). At 50% inhibition level, the difference between trend lines in vertical intercept manifests the relative allergenic potency. The analyzed data from response trend line equations show no significant difference between lots which confirm same IgE-binding properties and identical biological activity of the new house dust mite allergen batches.

*In vitro* allergen activity of the extracts can be measured by flow cytometry test for allergen-induced degranulation of basophils. We analyzed five A13 *D. pteronyssinus* batches by this flow cytometry method. The results from standardization of two last lots from house dust mite allergen using basophil activation test are present on **Fig. 4a**, and **Fig. 4b**. The percentage of degranulated basophils after activation with IHRP and new lots is very similar in each studied patient. These data correlate with the results from *in vivo* test – SPT and confirm indisputably inclusion of flow cytometry test in standardization procedure as a fast and reliable *in vitro* method for determination of total allergen activity.



**Fig. 4.** Comparison of allergen-induced basophil degranulation capability after activation with different lots from house dust mite allergen.

a) A13 D. pteronyssinus Lot № 01 2005 - OP

b) A13 D. pteronyssinus Lot № 01 2006 - OP

## Conclusions

Bulgarian house dust mite allergen (*A13 D. pteronyssinus*) is standardized by reliable procedure, which guarantees proper extraction, identity and similar total allergen activity from lot-to-lot. The data from the new *in vitro* methods for determination of the allergen's biological activity supplement and enrich the results of the accepted procedure for standardization. The introduction of these methods in our standard operation procedures for preparation and standardization of mite allergen will fulfill completely the recommendations of the European pharmacopoeia.

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