

Determination of the stability of diluted allergen extracts using a concentration step prior to EAST inhibition

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Summary

Background Generally the stability of diluted allergen extracts, as used for skin testing, provocation testing and immunotherapy can not be measured using a normal enzyme allergosorbent test (EAST) inhibition method.

Objective The aim of this study was to determine the stability of diluted allergen extracts using an ultrafiltration step prior to the standard EAST inhibition procedure, in which the allergen extract was concentrated 100-fold.

Methods This concentration procedure was validated for *Dermatophagoides pteronyssinus*, timothy pollen, birch pollen and cat dander extracts and used in a stability study in which three batches were stored for 1 year at 6°C and 25°C.

Results There was no difference in relative potency before and after concentration of birch and timothy pollen extracts. *D. pteronyssinus* and cat dander extracts showed a significant decrease of 25% and 35% of the relative potency after concentration. The mean coefficient of variation of 12 determinations of the stability study was 11.8%.

Conclusion For all allergens the 30 BU/mL or approximately 0.00025 mg/mL solution was stable for 12 months at both temperatures, except for *D. pteronyssinus* which declined rapidly at 25°C.

Keywords: birch pollen, cat dander, *D. pteronyssinus*, EAST inhibition, intracutaneous test solutions, stability, standardized allergens, timothy pollen

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Introduction

According to a recent position paper on skin testing [1] a prerequisite for scientific and routine diagnostic skin testing is the use of standardized allergen preparations, i.e. with known composition, potency and stability. This is also true for allergen preparations used for bronchoprovocation and immunotherapy.

Although documented stability of allergen extracts is important for reliable results of diagnostic preparations for *in vivo* tests, published research in this area is limited.

The preservative phenol has been reported to be destructive to aqueous pollen extracts [2]. This effect could be prevented by adding the stabilizer human serum albumin (HSA) [3,4].

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Naerdal *et al.* [5] reported a 10 BU/mL *D. farinae* extract to be stable for 8 months in saline containing 0.03% human serum albumin (HSA), as determined with the intracutaneous test. No stability studies have been published concerning cat dander extracts to date. Diluted extracts were reported to be less stable than more concentrated extracts [3,4]. Weber [6] proposed that intradermal materials and immunotherapy extracts more dilute than 1:100 w/v should not be used beyond 3 months.

Relatively low concentrations of allergen extracts as used for intracutaneous testing, bronchoprovocation and/or immunotherapy can often not be determined using the conventional enzyme allergosorbent test (EAST) inhibition procedure.

The aim of this study was to test the stability of diluted allergen extracts using a concentration step prior to a standard EAST inhibition procedure. This procedure was

validated and used in a stability study of *D. pteronyssinus*, timothy pollen, birch pollen and cat dander extracts. The stability was determined after storage for 6 and 12 months at 6°C and 25°C of a concentration of 30 BU/mL.

Materials and methods

EAST inhibition

An EAST inhibition procedure was used for the determination of the potency of intracutaneous test (ICT) solutions. The ICT test solution was concentrated in duplicate a 100-fold, prior to the normal EAST inhibition procedure. One hundred millilitres of the extract is concentrated to a volume of 5 mL using a stirred ultrafiltration cell with a 8050 ultrafiltration membrane YM3 (Amicon, Beverly, USA) and further concentrated to approximately 1 mL using a Centricon microconcentrator. All ultrafiltration membranes had a cut-off value of 3000 Da. The exact concentration factor was determined by weighing of the final volume.

This concentration procedure was validated for all allergens. Two millilitres of a solution containing freeze-dried extracts of *D. pteronyssinus*, timothy pollen, birch pollen and cat dander (concentration of 0.024, 0.051, 0.025 and 0.56 mg/mL = 3000 BU/mL), was divided in two parts. A 1 mL aliquot was used in a normal EAST inhibition procedure for determination of the relative potency. A second 1 mL aliquot was first diluted to 100 mL and concentrated according to the procedure described above. Thereafter the relative potency of both solutions was determined in a standard EAST inhibition procedure by comparing to an in house reference extract using pooled sera of patients with RAST class ≥ 3 against the different allergens. Paper discs were used for coupling of standardized allergen extract. Optimal concentrations for IgE binding were 0.2, 1.0, 1.0 or 0.02 mg/mL for timothy pollen, birch pollen, *D. pteronyssinus* or cat dander extracts, respectively. The β -galactosidase labelled anti-IgE was obtained from Kabi Pharmacia (Uppsala, Sweden).

The best-fit linear regression lines were calculated between 10 and 90% inhibition. Using Student's *t*-test ($P < 0.01$) the regression lines of the reference and samples were checked for parallelism.

The C_{50} value (μ L inhibitor at 50% inhibition) was calculated, using the equation of the regression line. This value was corrected for the concentration of the sample.

Finally, the mean relative potency and the coefficient of variation was calculated. The results were validated according to the guidelines described by Anderson and Baer [7]. The normal EAST inhibition method was also validated for every allergen, performing four triplicate assays on two different days.

Stability study

The procedure described above was used in the evaluation of the stability of three batches of standardized extracts of timothy pollen (*Phleum pratensis*), birch pollen (*Betula verrucosa*), *D. pteronyssinus* (1000 BU corresponds to 0.54 μ g Der p 1) and cat dander (1000 BU corresponds to 0.31 μ g Fel d 1) during storage for 1 year at 6°C and 25°C. The allergen extracts were dissolved in phosphate buffered saline (PBS) containing 0.03% HSA and 0.5% phenol at a concentration of 30 BU/mL (ALK Benelux BV, Groningen, The Netherlands), commonly used in intracutaneous testing (ICT) and filled out in DIN8R vials of hydrolytic glass type I (Aluglas, Uithoorn, The Netherlands). This concentration is comparable to 0.00025–0.0050 mg/mL.

At the start of the study the relative potency of each batch of freeze-dried extract was determined in triplicate using EAST inhibition. After 6 and 12 months of storage the relative potency was determined in duplicate using the EAST inhibition procedure after 100-fold concentrating the extracts.

Results

The mean CV of the standard EAST inhibition method (four triplicate assays on two days) was 11.6%, 14.1%, 8.8% and 18.8%, for *D. pteronyssinus*, timothy pollen, birch pollen and cat dander extracts, respectively.

In Table 1 the results of the validation of the concentration procedure are presented. There is no significant ($P < 0.05$) difference in relative potency before and after the dilution and re-concentration of birch and timothy pollen extracts. *D. pteronyssinus* and cat dander extracts showed a significant decrease of 25% and 35% of the relative potency after concentration. The coefficient of variation (CV) of this concentration method was 3.04%, 11.7%, 6.32% and 2.45% for *D. pteronyssinus*, timothy pollen, birch pollen and cat dander, respectively.

Results of the stability study using the modified EAST inhibition method are given in Fig. 1. The mean CV of 12 determinations was 14.5%, 12.9%, 9.4% and 10.2% for *D. pteronyssinus*, timothy pollen, birch pollen and cat dander, respectively.

All allergens were stable for 1 year at both temperatures, except for *D. pteronyssinus*, which was stable at 6°C, whereas the IgE binding activity declined rapidly at 25°C to 49% after 6 months and 18% after 12 months of storage.

Discussion

It is important to use standardized allergens with a documented stability for diagnostic skin testing, broncho-provocation testing and immunotherapy [1].

Table 1. Validation results of the concentration procedure

Allergen	Not concentrated			Diluted and concentrated		
	Results ¹	Mean	CV%	Results ¹	Mean	CV%
DP ²	1.04	1.00	5.56	0.76	0.75	3.04
	0.96			0.73		
Timothy	0.96	1.10	18.4	1.22	1.12	11.7
	1.24			1.03		
Birch	1.69	1.46	21.6	1.21	1.26	6.32
	1.24			1.32		
Cat	1.35	1.41	5.40	0.93	0.92	2.45
	1.46			0.90		

¹ Results expressed as relative potency. ² *D. pteronyssinus*.

The reproducibility of the standard EAST inhibition method is good and comparable or better than the mean intra-laboratory CV of 44% (2–100%), 45% (1–419%) and 48% (0–290%) reported for the RAST inhibition by Gjesing *et al.* [8], Arntzen *et al.* [9] and Ford *et al.* [10], respectively. The reproducibility of the concentration procedure was comparable to a standard EAST inhibition procedure.

After concentration, the total allergenic activity of cat dander and *D. pteronyssinus* was significantly lower. The reason for this decrease is not known, but is probably not caused by breakdown of the allergens or leakage of allergens through the ultrafiltration membrane as the used membrane had a cut-off value of 3000 Da. For practical

purposes this means that for *D. pteronyssinus* and cat dander a decrease in allergenic activity that is significantly lower than 25% or 35% would imply a significant breakdown of allergens.

After the validation this method was used in a stability study using the low concentration of 30 BU/mL of four allergen extracts. The mean CV of 12 determinations was good and also comparable to a normal EAST inhibition procedure.

Center *et al.* [2] reported that an aqueous ragweed pollen extract lost 50% of the antigen E content within 5 months of storage at 4°C due to the destructive effect of phenol in the preparation used. This effect was prevented by adding HSA to diluted pollen extracts, although some degradation occurred at the lower concentrations [3,4]. Naerdal *et al.* [5] reported that diluted aqueous mite preparations could be stabilized by the addition of HSA to the phenol-saline buffer, extending the stability to at least 8 months at 4°C. This effect is probably caused by prevention of adsorption of the allergens to glass surfaces. In a report, Weber [6] stated that even with the addition of HSA to phenolated saline solution an appreciable loss in activity occurred between 3 and 12 months. Therefore, it was proposed that intradermal materials and immunotherapy extracts more dilute than 1 : 100 w/v should not be used beyond 3 months. Our results are in accordance with the findings of Center, Naerdal and Nelson, but do not agree with the conclusions of Weber. This could be related to the protein content of the used extracts, as it was reported that preservation is related to the total protein content of the extract [4,11]. Extrapolation of stability results obtained with EAST inhibition to *in vivo* activity of the allergen extract should be done with caution, because discrepancies between *in vitro* and *in vivo* tests have been reported [12,13], indicating a detection of

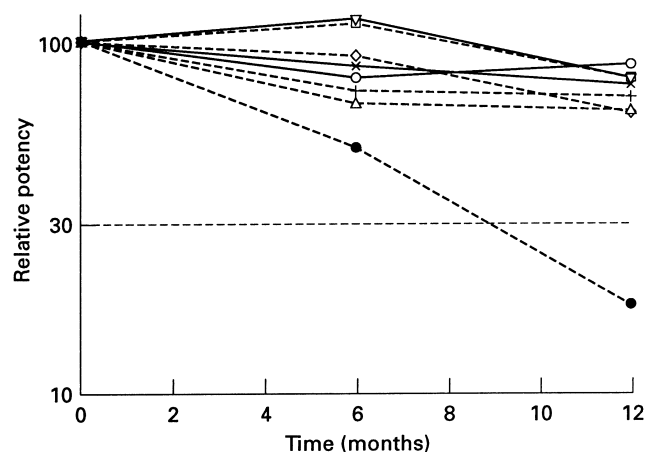


Fig. 1. Results of the stability study for timothy pollen at 6°C (○) and 25°C (+), birch pollen at 6°C (▽) and 25°C (□), *D. pteronyssinus* at 6°C (△) and 25°C (●) and cat dander at 6°C (×) and 25°C (◇), as measured by modified EAST inhibition for intracutaneous test concentrations at 30 BU/mL.

breakdown using EAST inhibition, whereas the skin reactivity was unchanged. However, no reports were published thusfar in which the skin reactivity was decreased, whereas the EAST inhibition results were not changed.

Another possibility of determining the stability of allergen extracts, could be the assay of single allergens (e.g. Der p 1). This method is not a total potency determination, but determines the concentration of only one allergen. Further, this assay is not available for all allergens. On the other hand this method would be able to measure the allergens directly without needing a concentration step.

From this study it can be concluded that diluted allergen extracts, as used in ICT, bronchoprovocation and immunotherapy, when stabilized with HSA, stored in vials of hydrolytic quality I can be stored at 6°C for at least 12 months without significant loss of IgE binding activity.

Further, it can be concluded that this method causes a significant decrease of the total allergenic activity for cat dander and *D. pteronyssinus*. However, this decrease was relatively small and is irrelevant to some biological applications (e.g. skin test), while for other applications this may be of importance. With the described limitations this method can be used for the determination of very low concentrations of allergen extracts (30 BU/mL or approximately 0.00025 mg/mL).

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