Fractionation of Source Materials Leads to a High Reproducibility of the SQ House Dust Mite SLIT-Tablets

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Abstract

Background: The production of house dust mite (HDM) allergen products for allergy immunotherapy has traditionally been based on purified mite bodies or whole-mite culture, which are quite different source materials with a limited possibility for adjusting the chemical composition. The SQ HDM SLIT-tablet is a fast-dissolving pharmaceutical formulation that has been developed for sublingual immunotherapy (SLIT) of HDM respiratory allergic disease. Objective: The objective of the present study was to establish a process for the production of drug substances for the SQ HDM SLIT-tablet offering a high reproducibility and independent control of the major allergens. Methods: Process controls were documented in a comprehensive process parameter qualification. The analyses comprised composition by crossed immuno-electrophoresis, protein content by BCA, total IgE binding potency by Centaur assay, quantitative major allergen determination by radial immunodiffusion and ELISA, and the ranking of emPAI scores generated by mass spectrometry. Results: Analysis of 20 batches of final product yielded a normalized mean and standard deviation for IgE binding potency of 100 ± 4.5. The standard deviation in the contents of Der f 1 and Der p 1 were correspondingly 11.9 and 6.1, whereas the variation in the group 2 major allergen content was 6.4. All measured 95% confidence limits between batches were less than 12%. Conclusions: The production process for the SQ HDM SLIT-tablet based on the separation of source material into four fractions each enriched in one major allergen enables precise adjustment of the relative major allergen content and high reproducibility of the final product.

Introduction

The prevalence of respiratory allergic disease is increasing and it is predicted that within the next few decades more than half of the European population will suffer from some type of allergy [1]. Respiratory allergic disease affects patients’ quality of life [2]. It reduces work and school performance and poses an economic burden to societies and health care systems [3]. Allergy is a chronic disease with a large risk of aggravation in symptom severity, such as rhinoconjunctivitis developing into asthma, and the risk of acquiring new allergies increases with the
number of allergies already present [4]. Many patients treated with pharmacotherapy report residual symptoms [5], and there is a need for additional treatment options.

Allergy immunotherapy (AIT) is the only allergy treatment with disease-modifying potential [1]. AIT is performed by repeated administration of standardized allergen products for the purpose of inducing immunological tolerance.

Respiratory disease caused by house dust mite (HDM) allergy is highly prevalent [6]. HDMs infest human habitats, where they feed on human skin scales and thrive particularly well in bedding. Mites, mite fragments and fecal pellets become airborne, and HDM-derived particles carrying allergens are inhaled. HDM allergens sensitise susceptible individuals and cause allergic symptoms in already sensitized individuals [6].

Products for HDM AIT are manufactured from aqueous extracts of raw materials produced by growing the dominating mite species, *Dermatophagoides farinae* and *D. pteronyssinus*, in pure cultures. Traditionally, raw materials have comprised either whole-mite culture or purified fractions, such as fecal particles or mite bodies [7]. Different raw materials and production processes are used for the production of HDM allergen products, and products from different manufacturers differ in composition and clinical performance [8, 9].

More than 20 HDM allergens have been characterized and recognized by the IUIS Allergen Nomenclature Subcommittee (www.allergen.org); however, group 1 and 2 allergens seem to be of particular importance in terms of IgE prevalence and potency [10]. The group 1 allergen is a cysteine protease, a digestive enzyme found in high amounts in fecal particles [11], and the group 2 allergen is a carrier protein found primarily in mite bodies [12].

The recent introduction of sublingually administered AIT tablets (SLIT-tablets) inaugurate a new era for immunotherapy. SLIT-tablets are being developed in comprehensive clinical development programs designed to fulfill requirements for regulatory approval [13, 14]. The basis for these developments is the establishment of robust and controlled production processes. In this article we describe a high reproducibility, including independent control of the important major allergens of HDM drug products, for a new SQ HDM SLIT-tablet (ALK, Hørsholm, Denmark). Randomized, double-blind, placebo-controlled trials documenting the efficacy of the SQ HDM SLIT-tablet described here in the treatment of adults and adolescents with HDM respiratory allergic disease have been reported elsewhere [15–17].

**Materials and Methods**

**HDM-Allergic Patients**

Thirty HDM-allergic patients who participated in the MT-02 trial [15] were selected for the crossed radio-immunoelectrophoresis (CRIE) analysis described below. Selection criteria included a history of HDM allergy, positive skin prick test and specific IgE of class 2 or higher. Sera were selected to represent low, intermediate and high IgE responses to HDM. For the total allergenic activity (see below), a pool of serum was prepared by mixing sera from 10–15 HDM-allergic patients. Individual sera were class 3 or higher (>3.5 kU/l IgE) to HDM, and reacted with one or more major allergen and different subsets of minor allergens. Sera were negative (≤0.35 kU/l IgE) to carbohydrate determinants, bovine serum albumin, milk proteins and gelatin. Selection criteria followed the EMA guideline on allergen products [18]. The study was approved by the Committee for ethics of science in the Copenhagen area (record No. H-3-2011-160).

**Crossed Immunoelectrophoresis**

The antigen profile was assessed by crossed immunoelectrophoresis (CIE) performed as previously described [19]. Allergen extract was applied to an agarose gel and separated according to size and charge by applying an electric field. After the separation of proteins, electrophoresis was continued at an angle perpendicular to the first, forcing proteins to migrate into an agarose gel containing polyclonal rabbit antibodies raised by immunizing rabbits with *D. farinae* and *D. pteronyssinus* antigens, respectively.

**Crossed Radio-Immunoelectrophoresis**

The antigen profile was assessed by CRIE performed as previously described [19]. CIE plates were incubated with serum followed by washing and incubation with biotinylated anti-human IgE. Subsequently, plates were washed and incubated with 125I-labeled streptavidin. Plates were finally washed and dried and subjected to autoradiography for 1, 3 and 10 days, enabling semiquantitative assessment based on the scoring of timing and intensity of staining of the X-ray films [20]. The scoring of films was performed in duplicate by the independent assessment by two researchers.

**Major Allergen Content by Radial Immunodiffusion**

For the assessment of source materials and drug substance the contents of the major allergens (Der f 1, Der f 2, Der p 1, Der p 2) were determined by radial immunodiffusion (RID; Mancini technique) using monospecific polyclonal rabbit antibodies. Four different antisera were prepared by the immunization of rabbits using preparations of purified major allergens. The major allergens were purified by biochemical methods combining techniques such as ion exchange chromatography, hydrophobic interaction chromatography and size-exclusion chromatography. Calibration curves were constructed using an in-house reference preparation (IHRP) of *D. pteronyssinus* and *D. farinae*. Sample responses in RID were directly correlated to the major allergen concentration in the samples with a precision below the 10% coefficient of variation and a coefficient of correlation for linearity at 0.97–1.00.

**Major Allergen Content by ELISA**

For assessment of the final drug product three validated sandwich ELISA [21] analytical methods were utilized to determine the
Der f 1, Der p 1 and group 2 major allergen content. Group 2 major allergen content was determined using a monoclonal antibody with a similar affinity for Der p 2 and Der f 2 used to quantify the combined content of these two major allergens (data not shown). The ELISA methods, including the production of the monoclonal antibodies, were performed essentially as previously described [22]. A calibration curve using an IHRP was established and major allergen activity was interpolated on the calibration curve.

**Total Allergenic Activity by Centaur**

The total allergenic activity was determined using an automated Centaur assay [23], applying a pool of human serum from HDM-allergic patients (see above). The assay employed a reverse sandwich architecture using monoclonal mouse anti-human IgE antibody covalently bound to paramagnetic particles in the solid phase (Siemens, Ballerup, Denmark). Bound sIgE reacted with a small amount of soluble biotinylated allergen mixed with dilutions of the sample under investigation. The read-out was the number of chemiluminescent light emissions upon excitation of acridinium ester-labeled streptavidin (Siemens). The measured luminescence was inversely correlated to the amount of allergen in the sample/reference. The allergenic activity was determined relative to the IHRP in the validated assay.

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**Fig. 1.** Process diagram: production processes for the SQ-HDM SLIT-tablet. Quality controls are indicated below the diagram.
Mass Spectrometry

The sample based on the drug substance was reduced with di-thiothreitol, alkylated with iodoacetamide and digested overnight with trypsin. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses were performed on an Ultimate 3000 RSLC nano System (Thermo Fisher Scientific) with nano flow using a C18 pre- and analytical column in line with the MS (maXis®; Bruker, Billerica, Mass., USA). Data were analyzed with Data Analysis v.4.0 (Bruker), BioTools (Bruker) and Mascot (Matrix Sciences). Allergens were identified by searching the MS/MS spectra against an in-house allergen database including published HDM allergen sequences. The ranking of individual allergen molecules in different batches was based on the semiquantitative EMPAI score resulting from the mascot search [24].

Results

A process for the production of HDM drug substances for the SQ HDM SLIT-tablet was established, including comprehensive measures for quality control. The SQ HDM SLIT-tablet is a freeze-dried unit containing aqueous allergen extract in a gelatin matrix, representing a mixture of the two dominating indoor HDM species, *D. farinae* and *D. pteronyssinus*. This report is focused on the consistency and reproducibility of the production process, featuring separation of the source material into four fractions, each enriched in one of the four major allergens, Der f 1, Der f 2, Der p 1 and Der p 2 (fig. 1).

Fig. 2. Diagram of the mechanical sieving process.

Fig. 3. Particle size of processed source materials. a Dried whole-mite culture. b Fraction containing primarily mite bodies. c Fraction containing primarily fecal particles. Original magnification ×51.2.
Production Process in Brief

Production of Raw Material

The HDM species *D. farinae* and *D. pteronyssinus* were cultivated separately under controlled temperature and humidity conditions, and then killed by freezing at –20°C. Cultures were verified to be devoid of live mites by visual inspection before drying in a fluid bed dryer with controlled air flow and temperature to a moisture content below 15%.

Fractionation by Mechanical Sieving

Whole-mite cultures were mechanically separated into four fractions using an automated sieve unit (fig. 2). The first fraction containing the largest particles above 350 μm constituted mostly gross medium and was discarded. The second fraction containing particles in the range between 90 and 350 μm was primarily mite bodies and was saved for subsequent extraction. The third fraction containing particles in the range between 50 and 90 μm was mostly mite parts and fecal agglomerates and was discarded, whereas the fourth fraction with particles below 50 μm contained primarily mite fecal particles and was saved for subsequent extraction (fig. 3). Fractions enriched in mite bodies and fecal particles were extracted separately and processed further to form the drug substance. The release criteria for active raw material frac-

![Graphs showing volumetric purity](image)

**Fig. 4.** Volumetric purity. The graphs show the percentage of particles observed under the microscope: fractions containing primarily fecal particles (a, b); fractions containing primarily mite bodies (c, d). Filled circles = Fecal particles; filled squares = bodies; open circles = fecal particles; crosses = medium; filled triangles = contaminating particles.
tions were based on the following quality control: identity and visual appearance, and microscopic counting of mite bodies (>90%), fecal particles (>95%) and contaminating particles (<1%; fig. 4).

Drug Substance
Each of the four extracted fractions was processed into an intermediate product, which was stabilized as frozen droplets (fig. 1). Frozen droplets represent a stable intermediate which can be dispensed by weight, and they were generated by the passage of the intermediate product through a nozzle. The process was controlled by the assessment of major allergen content after stabilization (data not shown).

Fractions containing purified mite bodies or fecal particles were extracted in aqueous buffer before the segregation of insoluble material by cross-flow filtration. Extracts were subsequently concentrated by ultrafiltration before being filtered through a 0.22-μm filter.

For each of the two mite species, *D. farinae* and *D. pteronyssinus*, one drug substance was produced by mixing intermediate product (frozen droplets) derived from mite bodies and mite fecal particles in a 1:1 ratio (w/w) based on the content of major allergen (data not shown). It should be noted that a 1:1 ratio based on weight corresponds to a 1:2 (Der 1:Der 2) molar ratio.

Final Drug Product
The two stabilized drug substances based on *D. farinae* and *D. pteronyssinus* were mixed 1:1 based on a standardized potency measure calculated as a combination of IgE binding potency and assays for group 1 and 2 major allergens. Excipients, gelatin and mannitol were added to form the drug product, and dispensed into blister pockets and freeze dried. The blister cards were sealed with aluminum foil. Gelatin and mannitol were added to provide structure to the tablets.

Quality Control
The active ingredient in an allergen product is the proteins. All known allergens are proteins and any protein is a potential allergen; therefore, quality control is particularly focused on the protein components. Batch-to-batch standardization of allergen products is performed through the comparison of every batch with an IHRP using scientifically based laboratory methods.

In-House Reference Preparation
An IHRP was established based on the new production process for each of the two mite species. The antigen profiles of the IHRPs were assessed by CIE and the allergen profiles by CRIE. In total, 44 antigens and 24 allergens were identified in the analyses using the drug substance derived from *D. farinae* (fig. 5a), whereas 53 antigens and 28 allergens were identified in the drug substance derived from *D. pteronyssinus* (fig. 5b). Allergens were identified by staining in the CRIE experiments using serum from 30 HDM-allergic patients.

Mass Spectrometry
A relatively crude application of LC-MS/MS was used to identify and rank the allergens present in the HDM drug substance. Samples were digested with trypsin and the resulting peptides separated by reverse-phase chromatography. The eluting peptides were sprayed directly into an ESI-QTOF mass spectrometer. Allergens and emPAI scores were identified by searching the MS/MS spectra against an in-house allergen database. The resulting semi-quantitative emPAI scores were used to rank the identified allergens, and the batch consistency was evaluated by statistical analysis of the rankings from 16 batches of *D. farinae* and 10 batches of *D. pteronyssinus* [24]. There was a statistically significant correlation between the ranking of individual allergens in different batches (data not shown), indicating a consistent batch composition.

Routine Batch Analyses, Drug Substance
Routine batch quality control includes assessment of the dry matter, protein content, protein profile, allergen profile, IgE binding potency and major allergen content (fig. 1). Results from the analysis of 20 batches of *D. farinae* and 24 batches of *D. pteronyssinus* are included in this study (table 1).

Based on the 20 and 24 batches of drug substance, the standard deviation in the variation of the content of dry matter relative to the mean was 7.7 for *D. farinae* and 6.8 for *D. pteronyssinus*. The corresponding figures for protein content were 13 and 11 (table 1).

The integrity of the antigen and allergen profiles was assessed by CIE and CRIE, respectively. The result of the CIE experiment is scored by subjective comparison of the pattern in the new batch with the IHRP. Each antigen immunogenic in rabbits forms a bell-shaped precipitate with a characteristic morphology and horizontal position, and the area is proportional to the amount. The test result is semiquantitative and a similar pattern thus indicates an identical composition. Small deviations between the batch and the IHRP are acceptable, for example small differences in distinctness or in area of specific precipitates.
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Fig. 5. CIE (upper panels) and CRIE (lower panels) of the drug substances based on D. farinae (a) and D. pteronyssinus (b). HIRP images are to the left; images to the right depict a random sample.

Table 1. Batch-to-batch consistency, drug substance

<table>
<thead>
<tr>
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<th>D. farinae (n = 20)</th>
<th>D. pteronyssinus (n = 24)</th>
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<tbody>
<tr>
<td></td>
<td>norm. mean (SD)</td>
<td>95% CI</td>
</tr>
<tr>
<td>Dry matter</td>
<td>1.000 (0.077)</td>
<td>0.96; 1.04</td>
</tr>
<tr>
<td>Protein content</td>
<td>1.000 (0.133)</td>
<td>0.94; 1.06</td>
</tr>
<tr>
<td>IgE binding potency</td>
<td>1.000 (0.129)</td>
<td>0.94; 1.06</td>
</tr>
<tr>
<td>Group 1 major allergen</td>
<td>1.000 (0.117)</td>
<td>0.95; 1.06</td>
</tr>
<tr>
<td>Group 2 major allergen</td>
<td>1.000 (0.123)</td>
<td>0.94; 1.06</td>
</tr>
</tbody>
</table>

All parameters were normally distributed. Data were normalized relative to the mean and listed here as the mean, standard deviation (SD) and 95% confidence interval (CI).
Table 2. Batch-to-batch consistency, final drug product (n = 20)

<table>
<thead>
<tr>
<th></th>
<th>Norm. mean (SD)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE binding potency</td>
<td>1.000 (0.045)</td>
<td>0.98; 1.02</td>
</tr>
<tr>
<td>Der f 1 major allergen</td>
<td>1.000 (0.119)</td>
<td>0.94; 1.06</td>
</tr>
<tr>
<td>Der p 1 major allergen</td>
<td>1.000 (0.061)</td>
<td>0.97; 1.03</td>
</tr>
<tr>
<td>Der 2 major allergen</td>
<td>1.000 (0.064)</td>
<td>0.97; 1.03</td>
</tr>
</tbody>
</table>

All parameters were normally distributed. Data were normalized relative to the mean and listed here as the mean, standard deviation (SD) and 95% confidence interval (CI).

The standard deviation in the HDM-specific IgE binding potency relative to the mean was 12.9 for *D. farinae* and 11.1 for *D. pteronyssinus* (table 1). The variation in the major allergen content measured by RID for group 1 was 11.7 for *D. farinae* and 9.0 for *D. pteronyssinus*. The corresponding figures for group 2 were 12.3 for *D. farinae* and 9.9 for *D. pteronyssinus* (table 1). Thus, for all the drug substance parameters measured, the variation between batches was less than 15%.

Routine Batch Analyses, Drug Product

Quality analysis of the final product included assessment of the IgE binding potency by Centaur assay and major allergen content by ELISA. The analysis of 20 batches yielded a normalized mean and standard deviation for IgE binding potency of 100 ± 4.5. The standard deviation in the contents of Der f 1 and Der p 1 were correspondingly 11.9 and 6.1, whereas the variation in the group 2 major allergen content was 6.4 (table 2). All 95% confidence limits on variation between the final product batches were less than 12%.

The absolute amount of major allergen in the drug product was 0.6 μg per major allergen per development unit (DU). For the 12-DU tablet, which was recently documented in a chamber provocation study [16], and in a large confirmatory phase III trial [17] in patients with allergic rhinoconjunctivitis caused by HDM, this corresponds to roughly 15 μg of Der 1 (Der f 1 and Der p 1 combined) and 15 μg of Der 2 (Der f 2 and Der p 2 combined) per tablet.

Discussion

Allergen products are made from natural source materials and the control of natural variability is crucial for standardized high-quality products. High-quality products are necessary to support a more general use of SLIT-tablets facilitating self-treatment at home, and are executed by robust and validated production processes with integrated quality assessment of all relevant parameters.

In this study a new production process was established based on fractionation of the HDM source material into fractions enabling control of precise dosing of each of the four important major allergens in the finished product (table 2). The antigen profile analyzed by CIE showed the presence of 44 and 53 antigens for *D. farinae* and *D. pteronyssinus* (fig. 5). This is in alignment with results from previous studies, such as the Danish Allergen Standardization 1976 study, in which 51 antigens were demonstrated in a *D. pteronyssinus* extract [26]. These results show that the separate purification of bodies and fecal particles does not compromise the complexity of the final drug product.

The CIE technique is semiquantitative as the size of the bell-shaped precipitates is proportional to the amount of antigen applied to the gel. By comparing precipitate patterns with IHRPs run in parallel, routine batch-to-batch control includes not only identity and complexity but also relative ratios of all relevant antigens in the product.

The CRIE analyses demonstrated the importance of group 1 and 2 major allergens. Both allergen groups showed a prevalence of reactivity among HDM-allergic patients of >80%, and a high proportion of patients showed moderate-to-high reactivity, particularly for group 2 allergens.

The optimal ratio between group 1 and 2 allergens with regards to clinical efficacy and safety is not established. Historically, allergen products for HDM AIT have been based on either whole-mite culture or purified fecal particles with high concentrations of group 1 allergens, or purified mite bodies with a more balanced content. Using the new production process described here, a constant ratio of 1:1 (w/w) can be reproduced.

In this study two different methods were used for major allergen determination, RID and ELISA. RID is the most precise method for samples with a medium-to-high allergen concentration, and this method was used in the processing of the drug substance. In the final product, however, the allergen concentration is relatively low, and in this case ELISA is the more precise method. Furthermore, the final product contains gelatin, which hampers the use of immunodiffusion. The results of the two techniques were consistent.

The MS experiments demonstrated a correlation in the ranking of identified allergens based on the semiquantitative emPAI score [24]. Apart from demonstrating the
presence of important HDM allergens, the consistency in the rankings supports the consistency of the composition of the drug product. LC-MS/MS can provide additional information on the composition of an allergen extract as compared to traditional immunochemical analyses, and with proper optimization the technique is potentially very useful as a tool in the quality control of allergen products. The analytical application mentioned here, however, was not optimized for identification purposes, and therefore could not be used to document the presence of all allergens.

Fractionation of source materials for the production of HDM allergen products facilitates a very high reproducibility of drug substances resulting from the new production processes. According to the revised Monograph on Allergen Products [27], requirements for variation in protein content and IgE binding potency must be within variation limits of 50–150%, and the major allergen content should be within a 50–200% variation interval [18, 27]. Previous studies on batch-to-batch consistency of another HDM SLIT-tablet stated a variability of ±30% in terms of allergenic activity, as well as group 1 and 2 major allergen contents [28]. The present study showed variability not exceeding ±15% for the routine production of batches for the SQ HDM SLIT-tablets.

The unique aspect of the production process described here is separation of the source material into fractions by mechanical sieving. By this method four fractions are isolated, each of them rich in one of the four important major allergens, yet with all antigens represented. The group 1 allergen is a dominating constituent of the fecal particles and the group 2 allergen is prominent in the body fractions for both of the two mite species. Having access to the four fractions separately ensures a precise and reproducible composition of the drug product by mixing the calculated amount of each fraction into the drug product. In conclusion, a process based on fractionation of HDM source material ensuring optimal standardization and reproducibility of the SQ HDM SLIT-tablet has been established.

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Disclosure Statement

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