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Preparation allergenic pollen extracts; the points should be considered to make high-quality products

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ABSTRACT

Atopic diseases have an increasing trend worldwide during the last two decades. Determining the main cause of allergic diseases, allergens, is the first step in managing and improving the issue, usually is done by Skin Prick tests (SPTs). Having allergenic extract in high quality is desired to perform a reliable SPT. Several parameters of extracts are considered including composition, stability, potency, preservation conditions, and unit definition. In this review, these factors have been explained pointing to factors might have profitable points or harmful drawback in the quality of allergen extracts.

KEYWORDS

Pollen; Allergen Extracts; Skin Prick Test; Standardization

Background

As our societies become more modernized; atopic diseases get an increasing trend worldwide, which include allergic rhino-conjunctivitis, allergic asthma, atopic dermatitis, and food allergies.[1] As the main cause is still unclear, the decreasing rate of infections and changes in life style and foods could be the main cause.[2,3] Meanwhile, many of other scientists believe that air pollution is one of the main causes of the increasing trend of allergic diseases.[4]

There is an extensive amount of research focused on this subject during last two decades. And they found, the best way to prevent allergic diseases is to avoid the main source of allergy.[5] Allergen exposure is recognized as an important environmental risk factor particularly in genetically predisposed individuals.[6] The diagnosis of the allergic diseases usually requires a detailed history, physical examination, but finding specific allergens for any individual is not possible without skin prick tests (SPTs) and/or radioallergosorbent test (RAST).[7]

SPT are simply performed by applying an allergen extract to the skin and then pricking it with an appropriate needle-like instrument.[8] In sensitized individuals, it results in the formation of a raised wheal surrounded by an erythematous flare within 15–20 min, indicating a positive test reaction.[9] *In vitro*-specific

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IgE also supports the clinical diagnosis and helps to guide the allergist in the management of allergic diseases, it is often used as a positive control.[10]

The accuracy of skin tests depends on the availability of well-characterized and consistent allergen extracts. In addition, these extracts are used in immunotherapy of allergy, the main approach for allergic diseases.[11]

There is a dramatic demnad for allergen standardization and characterization to perform standard allergy detection and/or allergy immunotherapy. Several efforts are in progress around the world to prepare allergen extracts that can be used independent to the diversity of geographical climates,[12] the presence of facilities in different tablatures[4] according to the people heritage, and their local aeroallergens.

Allergen extracts are complex mixtures of proteins and glycoproteins. Many factors in the extraction process and subsequent handling of allergens may affect the final composition, potency, and stability of allergen preparations. To ensure reproducible results in the diagnosis and therapy of allergic diseases, high-quality allergen extracts are essential.[13]

Allergen extracts were manufactured during all the previous years can be divided into two groups according to the presence of major allergens: standardized allergen and non-standardized allergens. Most of the routine SPT and consequently their aeroallergen extracts are not standardized like pollen of trees, and some of them that are standardized can be easily seen in Table 1.

In preparing high-quality extract, many parameters are always considered, including allergenicity, stability, purity and, consistency.[10]

Standardized allergen extracts require extra parameters including defined composition and total allergenic potency, but the main purpose of standardization is providing a reference for calibration of any extract for diagnostic and therapeutic use.[14,15]

Standardization is based on determinant major allergen content, which is correlated with skin test reactivity.[16] Applying standard extracts with assuring major allergen concentration results in calculating the effective dose of

Table 1. List of standardized allergenic pollen which are accessible and their major allergens.

Common Name	Scientific Name	Major Allergens
Bermuda Grass	*Cyndon dactylone*	Cyn d I
Kentucky Grass	*Poa Pratensis*	Poa p 1, Poa p 5, Poa p 9
MeadowFescue Grass	*Fstuca elatoir*	Fes e 1, Fes e 13, Fes e 4, Fes e 5
Orchard Grass	*Dactylis glomerata*	Dac g 1, Dac g 2, Dac g 5
Redtop Grass	*Agrotis alba*	Agr a 1
Prennnial Rye grass	*Lolium perenne*	Lol p 1, Lol p 2, Lol p 3, Lol p 5, Lol p 9
Sweet Vernal Grass	*Anthoxanthum odoratum*	Ant o 1
Timothy Grass	*Phleum pretense*	Phl p 1, Phl p5
Short Ragweed	*Ambrocia artemisiifolia*	Amb a 1, Amb a2, Amb a 3, Amb a 5, Amb a 6, Amb a 7

any allergen specially when immunotherapy vial is used. Therefore, all the new technical facilities are going to improve detection of the real amount of allergen and not total protein content. In the year 2001, an EU funded project, CREATE project, was started to support standardize allergenic extracts based on major allergens and suggest recombinant allergens as references to reveal the amount of major allergens.[17,18] Among all these allergenic pollens, only nine were standardized. The list of standard allergenic pollens and companies can be easily seen in Table 1.

If not possible, at least try to have a standard method to prepare high-quality and safe allergen extracts. Here, the entire concept of allergenic pollen extract and their standardization principals are reviewd (Table 2).

Standardization of source material

The first step usually considered in preparing allergenic pollen extracts is providing source material, which is pollens. Allergens may be present in airborne particles like pollens of plants including trees, grasses, or weeds.[19] These particles are complex materials when exposed to humans consequently show allergic condition. So to provide standard active allergens, raw and unprocessed material should be selected.[20]

Table 2. Summary of the standardization process is divided into four principles that are providing source material, proper extraction method, establishing an IHR, and measuring units.

Preparation source material
Considering variation: • Geographical climate • Seasons and days
Extraction Filtration in appropriate: • Time • Temperature • PH
IHRP Determining: • Chemical composition • Allergenic potency/activity • Stability during 2 years • Purity • Toxicity • Safety
Units and measurement Mg per ml of major allergens BU per ml International units

The collection of pollens usually is performed by several methods followed by grinding or different ways of pollen segregation from stigma. The pollen may be passed through different mesh sizes, irradiation, or by flotation to get cleaned.[14] Finally, pollens are dried under manageable conditions and stored at 20°C in sealed condition. The highest level of contamination with other species pollens or mold spores can be accepted up to 1%. Furthermore, they should be free from other parts of the obtained plant, at least 5% of the final weight can be accepted.[20] Variation in quantitative composition depending on season and location of growth and the year of harvesting, in order to achieve a relatively constant product, harvests from different years, seasons, and different farms, after complete characterization *in vitro*, should be pooled for final allergen extracts. For this, the checklist should be prepared for all pollens which is obtained by collection in nature or from cultivated fields.[21]

The selection of the source materials should be performed by considering collector qualifications, collection procedures, and specificity for relevant allergen sources should be considered reasonable measures must be employed to assure that and are appropriate to determine the identity and quality of the source materials. Botanical centers usually approve and certify the identity of pollen. Visual and microscopic data have to be recorded. The processing and storage of source materials should be performed to ensure that there are no irrelevant or harmful substances, including microbial organisms. Source materials should be fresh or stored in a manner that minimizes or prevents decomposition. Particular parameters of the collection, pretreatment, and storage are highly recommended to be recorded.[22,23]

Composition and allergenic activity

Determining the composition of allergen extracts can be done in two different approaches including Non-IgE-based methods and IgE-based method.[24]

In **Non-IgE-based methods**, the earliest method of standardizing allergen extracts was introduced by Noon; he arbitrarily designated Noon units.[20] This weight-by-volume (w/v) system has been extended to virtually all allergen source materials and is still one of the most widely used syetems in clinical practice.[25] In last decades of the twentieth century, it became obvious that most allergens were proteins, after that the new trend and one of the most important steps to standardize allergenic extracts was to determine the protein nitrogen content (PNU).[26,27] The assay has been standardized by the U.S. Food and Drug Administration and is routinely used by all U.S. manufacturers.[20]

The limitations of both the w/v and the PNU systems become apparent when one considers the fact that changing extraction conditions can lead to significant variability in the composition and potency of allergen extracts; most proteins in extracts are not allergens.[25,28]

Some other methods like ninhydrin amino acid assay, crossed immunoelectrophoresis (CIE), SDS-PAGE, and Isoelectric focusing in polyacrylamide gel can be used for semi-quantitative analysis of the protein composition of allergen extracts. Recent developments in molecular biology have made it possible now to establish simple methods to determine the allergenic content of allergenic extracts.[29] Despite simple methods to determine protein content, they are not sufficient to ensure allergenic potency or even allergenic contents. In addition, measurements and units are ambiguous. Furthermore, by using all sophisticated methods of protein determination such as amino acid analysis, one cannot guarantee consistency. So it can be vividly seen that there is a dramatic need for IgE-based methods and its significance cannot be ignored.[30]

Committee for medicinal products for human use (CHAMPS) published a guideline on allergen products: Production and quality issues in 2008 emphasize on size exclusion chromatography to determine the degree of polymerization for characterization and control of modified allergens.[31]

The discovery of IgE antibodies and the development of immunoassays that measure allergen-specific-IgE led to the use of RAST and enzyme-linked immunosorbent assay (ELISA) inhibition procedures in allergen standardization, which was the most important step in IgE-based method.[32] In these type of assessments, dilutions of allergen extracts are allowed to compete with the solid phase for binding to IgE antibodies from sera of a patient with specific allergy.[25] The assay results have been correlated with the overall biological potency in allergic subjects, but with the ability of the IgE to bind the allergen and the variability of the specificity and concentration of the IgE in sera from the selected patients. Immunoblotting is generally used to evaluate the composition of allergen extracts. This method can also be used to define major allergens of the frequent allergenic pollens.[15]

Methods based on polyclonal and monoclonal antibodies recognizing major allergens have been successfully employed to extract standardization. This approach is not dependent upon the variation in the specificity of patient's IgE antibodies but requires the measurement of most, if not all, relevant major and minor allergens in an extract.[33] If important allergens that are responsible for clinical symptoms in some patients are not measured, diagnostic and therapeutic efficacy may not be predictable. CHAMPS published a guideline on allergen products: production and quality issues in 2008 emphasize on size exclusion chromatography to determine the degree of polymerization for characterization and control of modified allergens.[31]

Establishing a control to standardize and batches consistency

Biological standardization is time-consuming and not cost-effective. Most manufacturers have used their own units and few of them have used the biological units as defined by the Nordic Guidelines or FDA to avoid comparing with each other from consumers. Although determination of the amount of major allergen by ELISA is simple and cost-effective, the biological relevance of major allergen content has not been established.[34] Furthermore, European commission established an allergen standardization project named "Development of Certified Reference Material for Allergenic Products and Validation Methods for their Quantification" (CREATE); the aim of the project was mostly evaluation of the potential use of evaluation of available ELISA assays for measurement of major allergens using the candidate certified reference material and were enrolled for some allergens.[35]

The accessibility of purified allergen references will enable the assignment of major allergen content in mass units to the internal reference preparations which are in use in different laboratories, manufacturers, allergen research centers, or control authorities.

On the other hand, the production process of allergenic extracts like Extraction and purification usually result in an intermediate product. Part of one of the first batches of an intermediate product can be kept as an in-house reference standard preparation (IHRP). The IHR should be enrolled to calibrate any production. When an IHRP has been selected, it is characterized by suitable methods. The basic studies such as potency and composition of any batch can be performed with considering IHRP.[36] Production process, including control of raw materials, potency, ptability, should be documented at appropriate temperatures as a label for recommendations for storage.[37]

The most problematic issue in allergen extracts and also marketing is that clinicians have difficulties in understanding differences and similarities between units used by manufacturers. This may cause confusion, so The IHRP must be completely characterized by *in vitro* laboratory methods to demonstrate an adequate complexity as well as an appropriate content of relevant major allergen(s).[38] The potency of the IHRP must be determined by *in vivo* methods, such as skin prick testing. Adjusting the allergen wheal size should be compared directly to histamine reference to avoid differences in technique and testing personnel. Therefore, the histamine reference was accepted, both in clinical trials and in clinical practice, to adjust results among batches in the same unit, and among different clinics and ideally the content of major allergens should be determined in absolute amounts. Furthermore, the IHRP should prove effective in clinical trials of specific allergy immunotherapy. Specific activities of the IHRs should be compared with international standards.[15]

Stability of allergenic activity

The minimal potency and allergenic activity or efficacy of the product till the end of shelf life should be maintained and provided by three batches of product at least.[39] Data obtained from only one batch or one product may be extrapolated to products derived from other batches or allergens of the same family contained in the same composition but it should be completely justified. Stability studies may use immunological methods by antiserum or other methods like immune-blotting RAST, and ELISA in any case; information about the stability of the allergen extract should be clear over time.[40] The final diluents have a key role in stability and maintenance of allergenic activity; normal saline or phenol had shown a tendency to loss of potency in some extract during the time, while it was proved that glycerin or human serum albumin (HSA) may protect allergenic activity.[38] Diluents also can be made with a combination of some including saline with 0.4% phenol with or without 0.03% HSA, or saline with 0.4% phenol with or without 10% glycerin. Since phenol is a bacteriostatic agent become highly interested in aqueous extract including normal saline and HSA solution to avoid contamination. But some allergen extract potency loss has been observed while using phenol-saline alone including grass and birch pollens extracts.[14] In all immunotherapy extracts except 50% glycerin diluents, that there is no need for phenol, it is significantly required in others. HAS is usually added to retard loss of potency, it can reduce the negative effect of phenol in many extracts. Furthermore, it might play an inhibitory role in adsorbing of protein content to the surface of vials. Glycerin is a bacteriostatic agent, and could also decrease proteolytic activity. Although it may cause injection pain in the site of SPT, it is the most favorite diluent. At the lower concentration like 10% percent, glycerin is less effective as a bacteriostatic agent but still has a protective effect for allergenic activity.[14] So, most of the recommendation which emphasizes previously on separating high protease-containing extracts from pollen extracts currently allowed mixing pollen extracts with some high protease-like cockroaches and mold extracts for immunotherapy vials in glycerin at a concentration above 20%.[13] It is that observed protease-mediated degradation of pollen extract can be inhibited in this way.[41] The shelf life of at least 12 months or longer is only acceptable.[42] This dates for allergenic extracts regard to stabilizer diluents; some other factors include the concentration, volume of storage vials, the presence of proteolysis enzymes; and other allergen constituents influence the shelf life and expiration date.[43] The concentration of allergen extracts also affect the stability, low concentration usually expires earlier, and loss expected potency.[14] There are limited data for particular maintenance concentration, especially for none standardized allergen extracts with weak potency. In this cases, the glycerin solution is the best option to protect allergenic activity.[44]

Purity

The standard operating process initiates with identifying and sanitizing entire area, may use some ventilation hoods for preparation by selecting an optimum location free of distractions, and limited exposure to high-risk contamination item or activity is suggested. Personal protective equipment like gloves, glasses, or shields can serve as a barrier to viral contamination.[13] Moreover, many automation system and software have been developed to guarantee the purity of extracts. Allergen extracts used for clinical diagnostic and immunotherapy may be contaminated with another allergen(s) and raise an unspecific immune response. The impurity of allergen extracts can occur as a result of a variety of different conditions of sample collection[40] since the collection of pollens without any contamination with other pollens is a difficult process[45] and it was observed that up to 98% pollens were contaminated with other pollens. Furthermore, the pre-extraction process (milling, defatting), extraction condition and buffers, post-extraction (filtration), and storage can also influence the purity.[46]

Other extraneous, possibly, harmful substances, such as those derived from microorganisms, should not be present.[29] Endotoxins which derived from the cell walls of gram-negative bacteria may be originated from the proliferation of bacteria during extraction or storing source material and are released after the death of cell may contaminate allergenic extracts during the preparation process.[47] Endotoxins have various effects including fever and leucopenia. In the large dose of endotoxins, diarrhea, hemorrhages particularly in the gastrointestinal tract, hyperglycemia, shock, or even death can occur.[48]

In addition, contamination of source material with mycotoxin may cause a notable reaction because of their toxic nature.[36] Mycotoxins are produced by specific molds during extraction and preparation and can contaminate other extracts like pollens during the process.[49]

While most of the contaminations originate from other pollens because of the same mean diameter, size, or colors, there were some methods that were developed to analyze the purity including. In the case of extracts, the gel diffusion and the skin test methods are comparable in detecting 0.2–1.0% of known pollen contaminant.[14] To establish SPT, it is necessary to use sensitive volunteers that can make it sometimes unfavorable.[50]

Infra-red spectrography of pollen grains and ultra-violet absorption spectra of pollen extracts developed had no valuable outcomes.[51] Immunoelectrophoresis and chromatography with Sephadex column fractionation methods usually give very complex patterns causing doubtful insight about the usefulness.[52]

Ouchterlony method which has smallest amount of contamination can be detected by this method and that the lowest contamination of one pollen with another can be detected certainly.[53] It experimented that the soluble antigens from 100 to 250 µg of pollen the extract. So, it can be one of the appropriate methods.[51]

Removing low molecular-weight substances may be performed by dialysis. Generally, this is accomplished by the use of dialysis with molecular-weight cutoff such as 3–5 KD to allow low molecular-weight material such as vasoactive amines, mycotoxins, herbicides, heavy metals, and pesticides, to pass through the dialyzer tubing and be replaced with purified water or interested buffer over time.[54]

Aseptic filtration is the sterilizing process where the purified extract is passed through a sterilizing-grade filter (usually a 0.2 µ porosity) into a sterile collecting vessel, especially it is highly necessary for Immunotherapy vials.[51]

Storage

After preparing the allergen extracts, all criteria related to enhancement stability including use of the best kind of preservatives and stabilizers, temperature, and optimum storage condition must be considered. The storage condition influences the allergenic activity. Routinely most of the allergen extracts are kept at 2–8°C.[39] Since the stability of diluted extracts is less than concentrates, users should notice to the type of used extracts as well as the expiration date of each vial.[55] Storing with high concentration before packaging is highly recommended. Period of storage for mixed extract should be limited to avoid loss of potency. All the facilities and equipment for pollen preparation separately or mixed better to assemble prior to start to have better protection for staff. [13] The potency and complexity of allergenic extracts should be checked regularly by SPT in comparison with freshly diluted extract and polyacrylamide gel electrophoresis, immunoelectrophoresis, ELISA, direct-Bind (Sandwich) ELISA, and competition ELISA, respectively.[38]

Batch-to-batch consistency

The batch-to-batch standardization can be performed by determination of allergen composition and quantification of specific allergens to the grantee that all essential and important allergens are present in constant ratios. Moreover, they should ensure that the potency of the extract is constant by quantification of allergenic activity.[42] Evaluating allergenic extracts composition may be ensured by immunoblotting crossed (radio) immunoelectrophoresis (CIE/CRIE), IEF, and even SDS-PAGE. CIE is often preferred due to the advantage of being a semi-quantitative technique. It works by

precipitation pattern and as a reproducible method can ensure the consistency between different batches.[56] In order to assess consistency via quantifying major allergen, quantitative immunoelectrophoresis (QIE) and ELISA were proposed. In addition to quantification of total allergenic activity, RAST or RAST inhibition assays are suggested.[57] In conclusion, allergen extracts should be to be matched in all aspects by each and every following batch and this could be served by IHRP.

Unit and measurement

The most important step in measurement and units for manufacturing or preparation allergen extracts SPT, for the first time, is the weal's diameter. It should be considered 3 mm in diameter adjusting to the highest concentration.[58] These may be measured by BU per ml concentration of major allergens (mg per ml) for biologically standard extracts.[58] Moreover, there are international Units and also national or laboratory standards/Units which are designed to calibrate allergenic preparations. They are labeled international units or other formats arbitrarily, like 1000 IU in each vial for international standard/units.[59] Although the International Units have not any direct biologic relevance unlike BU per ml, they are useful for comparing extracts and provide the method for clear documents (composition and constant). In Europe, there is only one national unit, the Nordic system, which is used by most of the companies. So that European regulatory authorities are accepted for company units.[60]

Conclusion

Allergen extracts standardization is a suitable way to ensure an appropriate preparation of SPTs and immunotherapy vials. In the standardization process, some points should be considered as providing source material firstly and then establishing IHR which is used to calibrate stability, composition, potency, purity, safety, sterility. Finally, the allergenic pollen extracts should be modified and should be selected for diagnostic and clinical use and measuring units that depend on the presence of measurable major allergens.

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