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Advances in the quantification of relevant allergens in allergenic extracts

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Summary

Relevant allergens are major contributors to the safety and efficacy of allergenic extracts used in allergen immunotherapy (AIT). As such, they should be accurately quantified, as recommended by the 2008 European guidelines on allergen products. Until now, the quantification of relevant allergens was mainly performed by using immunoassays (e.g. ELISA) that relying upon specific antibodies. Although antibody-based quantification is commonly used to assess the concentration of relevant allergens in allergenic extracts, results must be taken with caution in the light of the inherent limitations of such techniques. In the present study, we discuss how those limitations can be overcome by using comprehensive mass spectrometry-based techniques

Introduction

The European Medicines Agency (EMA) defines relevant allergens as "allergens causing a clinically relevant effect in a significant proportion of allergic patients". Because of their importance, the presence of relevant allergens in commercially available allergenic extracts should be demonstrated whenever possible, by "using appropriate methods such as antibody-based techniques or mass spectrometry", as recommended by the 2008 European guideline on allergen products (1). In practice, relevant allergens are most often quantified by using antibody-based techniques. As a matter of fact, a number of commercial or proprietary immunoassays have been successfully developed and implemented for measuring allergen content in natural sources or therapeutic products. However, the results obtained with such techniques cannot be considered absolute. Indeed, the relevant allergen concentration measured with antibody-based techniques in a given allergenic extract can vary substantially depending upon the following (2): the technique used, that is, either RadioImmunoAssay (RIA), Enzyme-Linked ImmunoSorbent Assay (ELISA), Radial ImmunoDiffusion (RID), or Rocket ImmunoElectrophoresis (RIE); the specificities and affinities of antibodies used; and the reference standard used, and the way it is calibrated.

Herein, we report the quantification of relevant allergens in a series of aqueous extracts used for allergen immunotherapy (AIT). We also discuss the relative advantages and limitations of techniques based on antibodies, as compared with mass spectrometry (MS), for the quantification of relevant allergens.

Materials and methods

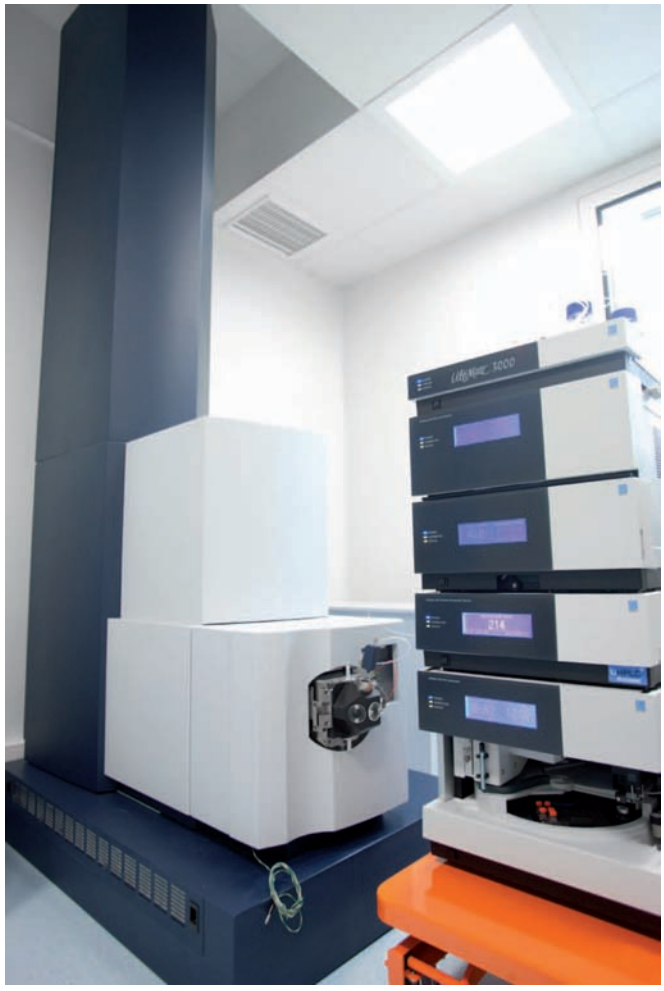
Antibody-based quantification of relevant allergens

For the quantification of house dust mite (HDM) Der p 1, Der p 2, and Der f 1, cat Fel d 1, dog Can f 1, and mould Alt a 1 relevant allergens, we used the corresponding sandwich ELISAs from Indoor Biotechnologies (Charlottesville, VA, USA), based either on the use of both polyclonal and monoclonal antibodies (Can f 1) or on the use of monoclonal antibodies (mAbs) only (other allergens). We used mAb-based sandwich ELISAs developed internally for the quantification of group 1- and group 5-relevant allergens from grass pollens, as well as of Bet v 1, Ole e 1, and Amb a 1 from birch, olive tree, and ragweed pollens, respectively. For the quantification of Jun a 1 from *Juniperus ashei* pollen, we used mAb-based quantitative western blotting.

Mass spectrometry (MS)-based quantification of relevant allergens

The extracts to be tested were spiked with a known amount of a reference standard, reduced with dithiothreitol, alkylated, and digested with trypsin prior to being analysed by liquid chromatography coupled to tandem MS (Figure 1). The amount of allergen in each sample extract was determined based on the amount of spiked reference standard. The method was fully validated and, as such, was shown to be both selective and linear (coefficient of correlation greater than 0.98), with both intra- and inter-run precisions below 15%.

Figure 1 - A liquid chromatography system coupled to a mass spectrometer



Results

Quantification of relevant allergens in aqueous extracts used for AIT

As measured by using antibody-based techniques, the amounts of relevant allergens in extracts used for immunotherapeutic purposes vary from 4 to 100 μg/100 IR, depending upon the allergenic extract considered (Table 1). Notably, such quantities of allergens obtained by immunoassays can only be interpreted in light of the technique and reagents used.

Advantages and limitations of antibody-based techniques

Immunoassays are broadly used to quantify allergens, as reliable and easy to implement techniques (Table 2). Nonetheless, such antibody-based assays face some limitations.

Firstly, the quantity of an allergen can vary according to the technique used (e.g. RIA as compared with an immunoelectrophoretic technique (2)).

Secondly, results obtained by immunoassays depend upon the way the standard used is calibrated, with respect to its protein concentration. For a given protein sample, the protein concentration can vary depending upon the method used to measure it (2). The only way to overcome this limitation is the use of the same standard by all manufacturers, such as the recombinant Bet v 1 and Phl p 5a, selected by the CREATE consortium, and established as a European Pharmacopoeia Chemical Reference Substance (4-8).

Table 1 - Relevant allergens in various allergenic extracts

Sources of allergenic extracts	Allergenic extracts	Relevant allergens	Quantities in μg corresponding to 100 IR*
HDM	*D. pteronyssinus*	Der p 1	20
		Der p 2	4
	D. farinae	Der f 1	50
Animal danders	Cat	Fel d 1	80
	Dog	Can f 1	20
Grass pollens	5-grass pollen	group 5	7
Tree pollens	Birch pollen	Bet v 1	50
	Olive tree pollen	Ole e 1	10
	Juniperus ashei pollen	Jun a 1	100
Weed pollens	Ragweed pollen	Amb a 1	100
Moulds	Alternaria	Alt a 1	6

* Average contents of relevant allergens in final extracts. 100 IR/ml (Index of Reactivity per milliliter) is the allergenic potency of an extract eliciting a mean wheal diameter of 7 mm in skin prick testing performed in patients allergic to the corresponding allergen source.

Table 2 - Advantages and limitations of immunoassays and MS-based techniques for the quantification of relevant allergens

Type of technique	Advantages	Limitations
Immunoassays: • RIE • RID • quantitative dot-blot and western blot • RIA • ELISA • multiplex miniaturized assays (3)	• Low cost materials and reagents; • Easy to implement; • Can be automated; • Antibody reactivity can be indicative of allergen stability, when associated with conformation changes or proteolytic degradation.	• Not always very sensitive (e.g. RID); • Not absolute; • Results depend upon the technique; the standard, and the specificities and affinities of antibodies; • Results cannot be compared between; laboratories, unless the same technique and the same reagents are used (4); • Stability of the antibodies has to be documented; • Depends to a non-measurable extent on the allergen conformation.
Mass spectrometry (MS)-based techniques, such as liquid chromatography (LC) coupled to tandem MS (MS/MS)	• Very high specificity, based on retention time in LC, the mass peptide obtained by MS, and the peptide sequence obtained in MS/MS; • High sensitivity; • Comprehensive quantification of the allergens (including all isoforms); • Can be fully automated; • Independent of allergen folding.	• Expensive instrumentation; • Need for an extensive characterization of the allergens, in order to determine the proteotypic peptides, i.e. the consensus peptides shared by all isoforms; • Not indicative of allergen stability.

Thirdly, the specificity of the antibodies used can also be an obstacle. This is directly linked to the remarkable diversity often observed with allergens from natural sources, which exhibit numerous isoforms and variants originating from primary amino acid sequence variations as well as from post-translational modifications. For instance, group 1 and group 5 allergens from grass pollens encompass 5–10 and 50–100 isoforms, respectively, for a given *Pooideae* species (9,10). Consequently, anti-allergen antibodies are sometimes "too specific", that is they do not detect all isoforms of the allergen to be quantified (11). As an example, characterization of the HDM Der p 2-relevant allergen purified from *D. pteronyssinus* extracts indicated that Der p 2.0101 is a prominent isoform of the allergen (12). This isoform is known not to be recognized by mAb 1D8 (13, 14), so that a Der p 2-quantification method based on this mAb fails to detect this isoform (15, 16). Similarly, we observed that mAb-based sandwich ELISAs often greatly underestimate the concentration of group 1-relevant allergens in grass pollen extracts, most likely because the mAbs used do not recognize all allergen isoforms (manuscript in preparation). With respect to this aspect, it is worth noting the following: (a) the sandwich ELISA developed by Duffort et al. for quantifying grass pollen group 1 allergens uses a mixture of three monoclonal antibodies (so-called "oligoclonal antibodies") for capture and polyclonal antibodies derived from rabbit for detection (17), thus likely encompassing most—if not all—isoforms; and (b) the European Union CREATE Project showed that three existing ELISAs for grass pollen group 1 allergen (namely Phl p 1), each based on two mAbs, do not allow satisfactory quantification of the allergen (18). Antibodies not only miss some isoforms of an allergen but they can also discriminate between different conformations of the same allergenic molecule (19).
Furthermore, the only way to overcome the limitation posed by the standard is the consistent use by all manufacturers of the same technique and the same reagents, such as the ones that could eventually be selected by the CREATE project for the quantification of Bet v 1 and Phl p 5a (4).

Allergen quantification using mass spectrometry (MS)

MS-based quantification has been identified as an attractive alternative for the quantification of relevant allergens (20, 21) (Table 2). If such an MS quantification is based on the detection of peptides that are conserved among all the isoforms of the allergen to be quantified, it can solve the issue of underestimated allergen concentrations due to reagents that are too specific. With the example of group 1 allergens from grass pollens, we have shown that MS-based quantification is applicable to allergens, as expected from what was documented previously with many other proteins (22-24). We confirmed that MS-based techniques allow a comprehensive quantification of natural isoforms of allergens as diverse as group 1 allergens from grass pollens and HDM Der p 2 (manuscript in preparation).

Discussion

Because of their importance and relevance, clinically relevant allergens should be accurately quantified in allergenic extracts intended for AIT. Stallergenes has acquired 20 years of experience in quantifying relevant allergens within natural extracts. The assays have evolved over time, from antibody-based methods to current approaches based on MS. Immunoassays are easy-to-implement techniques, but the allergen concentration obtained is strongly dependent upon the technique and the reagents used, which thus appears as the main limitation. Particularly, due to the structural variability of most natural allergens, immunoassays are often too specific, leading to an underestimated concentration of the corresponding allergen. This limitation can be overcome by using MS-based detection of peptides that are conserved among all the isoforms of the allergen to be quantified, thus allowing a comprehensive quantification of such allergens.
The use of the abovementioned techniques (i.e. antibody-based and MS-based techniques) leads to a further improvement of the quality of allergen extracts. These techniques are now applied all along the production process and constitute a pivotal and advanced step for the pharmaceutical characterization of the finished products in AIT, which are therefore considered as new and innovative drugs.

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