

Establishment of recombinant major allergens Bet v 1 and Phl p 5a as Ph. Eur. reference standards and validation of ELISA methods for their measurement

Results from feasibility studies

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ABSTRACT

The potency of allergen extracts is determined as total allergenic activity without consideration of their composition and the units differ from one manufacturer to another, making it very difficult to compare the different products. Recently, purified major allergens have been obtained by recombinant DNA technology and produced under Good Manufacturing Practice (GMP) conditions. In principle, such recombinant allergens could be established as reference standards and could help for the standardisation of the major allergen content of allergen extracts. Two recombinant major allergens, one from birch pollen, rBet v 1, and one from Timothy grass pollen, Phl p 5a, have been selected at the end of the CREATE programme as a potential starting point for the establishment as European Pharmacopoeia (Ph. Eur.) Reference Standards through a project run by the Biological Standardisation Programme (BSP) of the European Directorate for the Quality of Medicines & HealthCare (EDQM). To this end, bulk candidate recombinant materials, produced under GMP conditions, were procured from two European manufacturers and subsequently formulated and lyophilised.

Four ELISA systems from three different manufacturers were included in the project, two for Bet v 1 and two for Phl p 5a with the aim of establishing reference methods for determination of the respective major antigens both in natural allergen extracts as well as in recombinant allergen products.

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The project was run in 3 phases: a preparatory and preliminary testing phase (feasibility phase or Phase 1), an extended feasibility phase carried out in 3 laboratories (Phase 2) to confirm the transferability of the methods and an international collaborative study with a large number of participating laboratories (Phase 3). This article describes the work done in Phase 1 and Phase 2, i.e. the physico-chemical and biological characterisation of the recombinant candidate reference standards, the assessment of their suitability for the intended purpose as well as the evaluation of the candidate ELISA systems.

The results show that both candidate reference standards are suitable for the intended purpose. In addition, three out of the four ELISA systems that were included in the preliminary phase were found to be appropriate for further evaluation in the collaborative study which was organised in 2011. The results of the collaborative study will be published separately.

KEYWORDS

Allergen, major allergen, Bet v 1, Phl p 5, collaborative study, European Pharmacopoeia, reference standard, CRS, ELISA, biological standardisation.

1. INTRODUCTION

The European Union (EU)-funded multidisciplinary research project CREATE (Development of Certified Reference Materials for Allergenic Products and Validation of Methods for their Quantification) finished in April 2005. The major aim of this project was to introduce the measurement of individual major allergens to allergen standardisation and to identify appropriate reference materials for this approach [1, 2, 3]. It was attempted to achieve this through validation of ELISA systems for the quantification of major allergens in extracts, using defined reference materials as standards. However, the ELISA validation was not completed during the CREATE project. Furthermore, the establishment of a large batch of reference standard was outside the scope of the CREATE project.

During a meeting of the International Union of Immunological Societies (IUIS), Allergen Standardization Subcommittee, it was agreed that, based on the CREATE project, two major allergens, one from *Betula verrucosa* (birch) pollen (Bet v 1) and one from *Phleum pratense* (Timothy grass) pollen (Phl p 5a), and the corresponding ELISA systems from ALK-Abelló (Bet v 1 and Phl p 5a), Allergopharma (Phl p 5 a) and Stallergenes (Bet v 1) appeared to be the most promising systems for further validation.

A project proposal for validation of the ELISA systems and establishment of the reference standards (RS) was accepted by the Steering Committee of the Biological Standardisation Programme (BSP) at its meeting in January 2006 and the BSP090 project was started. In parallel, other activities in the field of allergen standardisation started. The Committee for Medicinal Products for Human Use of the European Medicines Agency revised the Note for Guidance on Allergen Products and the general monograph on allergens of the European Pharmacopoeia (Ph. Eur.) was also revised [4].

The project was planned to be run in 3 phases:

- an initial feasibility evaluation (Phase 1) in the laboratory of the project leader at the Paul-Ehrlich-Institut (PEI);
- an extended feasibility study with 3 participating laboratories (Phase 2);
- an international collaborative study (Phase 3).

Here we describe the results of the initial and extended feasibility studies (Phases 1 and 2).

The initial qualification phase in the project leader's laboratory, aimed at investigating the suitability of recombinant (r) Bet v 1 and rPhl p 5a as reference standards. Both allergens

were produced in recombinant form in *E. coli* under Good Manufacturing Practice (GMP) conditions. The rBet v 1 material was provided by Stallergenes and the rPhl p 5a material by Allergopharma.

Before initiating the feasibility study, a few steps proved to be necessary to assess the suitability of the lyophilisation procedure optimised within the CREATE project [1, 2, 3]. In addition, preliminary recovery experiments on extracts spiked with different concentrations of the corresponding recombinant allergen and preliminary testing of ELISA performance (e.g. range, intra- and inter-assay precision) were performed. During Phase 1, the lyophilised materials were tested against non-lyophilised material (herein referred to as 'bulk') in order to check the impact of lyophilisation on their suitability. The candidate reference standards (cRS) were also used to prepare spiked model samples (i.e. extracts spiked with different concentrations of the respective recombinant allergen) for the extended feasibility phase (Phase 2). These model samples were then freeze-dried, together with unspiked extracts, with the lyophilisation procedure optimised in Phase 1.

The GMP batches of allergens were submitted to an extensive physico-chemical and structural characterisation. The formulated and lyophilised candidate materials, however, could not be properly characterised or quantified by physico-chemical methods due to interference of the formulation components, mainly human serum albumin (HSA), and needed to be further characterised in biological assays, i.e. basophil histamine release after passive immunisation using sera from allergic patients [5].

Quantification of major allergens not only requires appropriate reference materials, but also well-validated corresponding immuno-assays for their measurement with standardised protocols. Therefore, four candidate antibody-based sandwich ELISA systems were evaluated during the feasibility testing (Phase 1): two Bet v 1 ELISAs (from ALK-Abelló and Stallergenes) and two Phl p 5 ELISAs (from ALK-Abelló and Allergopharma).

2. AIM

The project BSP090, sponsored by the Biological Standardisation Programme (BSP) of the EDQM, was intended to establish on a large scale Ph. Eur. RS for the two recombinant major allergens Bet v 1, from birch pollen, and Phl p 5a, from Timothy grass pollen, as well as to validate reference ELISA methods for their quantification in mass units.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Extracts

Birch pollen and Timothy grass pollen extracts were provided by ALK-Abelló, Allergopharma and Stallergenes. They were tested by ELISA for their content in Bet v 1 and Phl p 5a and a pool of the individual extracts from all three manufacturers was produced for further investigations to even out differences between manufacturers and to provide a mixed extract with appropriate major allergen content.

3.1.2. Recombinant allergens

rBet v 1 (lot 6-2/Y0487)

The material was produced by Stallergenes under GMP conditions. It was provided as an aqueous solution with a nominal concentration of 3.17 g/L.

rPhl p 5a (lot PP5ar06007)

The material was produced by Allergopharma under GMP conditions. It was provided as an aqueous solution with a nominal concentration of 1.823 g/L.

The concentration of the bulk materials was determined by amino acid analysis under GMP conditions (see section 3.2.2). These concentrations, different from the nominal concentration obtained from the manufacturers of the bulk materials, were used for assigning the protein content for the cRS in Phase 3. For Phases 1 and 2, the concentrations provided by the manufacturers were used.

3.1.3. Pooled extracts

Prior to spiking, the extracts obtained from all three manufacturers were pooled in order to blind differences between manufacturers. Model samples for the ELISAs were prepared by spiking pooled birch pollen and Timothy grass pollen extracts with defined amounts of the cRS for rBet v 1 or rPhl p 5a respectively. This was necessary to mimic the biological matrix in which major allergens are measured, i.e. use of the ELISAs and the recombinant allergen standards for quantification of major allergens in allergen extracts.

3.1.4. ELISA materials

The different manufacturers provided specific ELISA reagents together with specific, previously validated protocols. The other common ELISA reagents were purchased from commercial sources. The protocols provided were adjusted to a general format for a better practicability for the next phases of the study. However, the procedures of the different ELISAs were not changed.

3.2. Methods

3.2.1. Formulation and lyophilisation of samples

The recombinant allergen reference materials, single extracts, pooled natural extracts and spiked model samples were formulated and freeze-dried following a procedure that had been found to be suitable during the CREATE project [1, 2, 3]. Samples were formulated in a sterile 0.9% saline solution containing 0.1% D-(+)-trehalose dihydrate and 0.2% human serum albumin (HSA). For the preliminary experiments the lyophilisation was performed in the laboratory of the project leader. The formulation buffer was prepared as a 2-fold concentrated solution and identical volumes of recombinant allergens or extract and formulation buffer were mixed. One millilitre aliquots of the samples were dispensed into sterile 15 mL Falcon tubes and frozen at – 20 °C before starting the lyophilisation procedure. For use in Phases 1 and 2, each cRS was formulated and lyophilised at a pilot scale. Approximately 100 vials were produced for each candidate reference preparation, i.e. 3 mL white glass vials were filled with 1 mL of formulated recombinant allergen and subsequently freeze-dried. After freeze-drying, samples were stored at – 20 °C until use. During the preliminary phase the recombinant allergens and the single extracts were tested before and after lyophilisation in all four ELISA systems. They were also compared with pre-existing formulated and freeze-dried samples of rBet v 1 and rPhl p 5a from the CREATE project. Further assays with pooled extracts and spiked extracts were performed during Phase 1 with the three selected ELISA systems.

3.2.2. Physico-chemical tests and quantification of protein content of the cRS

Both cRS for rBet v 1 and rPhl p 5a were investigated at the University of Salzburg as bulk material using similar physico-chemical methods [6, 7].

Quantification of protein content was done in duplicate under GMP conditions at M-Scan SA, Plan-les-Ouates, Switzerland. The method used was an amino acid analysis involving an acidic hydrolysis of peptide bonds. The amino acid products were then quantified using a ratio-based calibration curve generated from analysis of mixtures containing known concentrations of the expected amino acids.

3.2.3. Biological tests

Physico-chemical properties of the formulated and freeze-dried candidate materials could not be assessed due to HSA interference. Therefore, both CRS were tested in the stripped basophil histamine-release bioassay, as described previously [5], against 10 different sera pools that had been collected for the CREATE project from allergic donors in order to compare their immunogenic properties before and after the formulation and lyophilisation process. Briefly, basophils are purified from buffy coats, stripped of their immunoglobulin E (IgE) by incubation with a lactic acid buffer, sensitised by human serum from allergic donors and stimulated with the allergen, either rBet v 1 or rPhl p 5a, with or without interleukin (IL)-3. The incubation with the allergen leads to histamine release, which is then measured by fluorometric analysis.

3.2.4. ELISA systems

Standard Operating Procedures (SOPs), specific for each system, had been optimised in previous experiments by the manufacturers and were obtained together with the necessary specific reagents (mainly antibodies, see section 3.1.4) although not as complete kits but rather as sets of antibodies or pre-coated plates (in the case of the ALK-Abelló systems). A list of additional commercially available reagents used by the manufacturers in their laboratories was also included. For each ELISA, the reagents were used as stated in the manufacturers' protocols, resulting in deviations, e.g. for incubation buffer or detection systems, between the different protocols.

Briefly, in general for each independent ELISA system, 96-well microtitre plates were coated with allergen-specific antibodies, at a concentration optimal to each system. Plates were then blocked and incubated in duplicate with serial dilutions of references and samples. Detection and colour reaction were carried out under appropriate conditions. The concentration of the samples was obtained by interpolation with reference dose-response curves. System suitability was checked by concomitant incubation of positive controls.

Reproducibility and precision of the different ELISA systems were tested on respective bulk and formulated/lyophilised materials. For measurement of intra-assay precision, one appropriate dilution of the corresponding mixed extract was spiked with 4 different concentrations of the corresponding CRS within the range and tested each with 6 replicates. To assess the intermediate precision (inter-assay precision), the assay was repeated on three different days. The range and limits of detection and quantification (LoD and LoQ respectively) were assessed by preparing serial dilutions of reference preparations as well as negative controls in the corresponding ELISA buffers. Mean concentration, coefficient of variation and mean recovery were calculated for each point to determine the LoQs and the range for each assay. The accuracy and linearity were measured by the standard addition method. Recovery experiments were performed with formulated and freeze-dried extracts and reference preparations as well as with bulk materials.

Allergen extracts used for skin prick testing are normally prepared using a buffer containing 0.9% NaCl, 50% glycerol and 0.5% phenol. The robustness of the assays was assessed by two different methods to evaluate the putative effects of such a buffer matrix. The rate of recovery was determined and compared to the rate of recovery of reference materials dissolved in corresponding ELISA dilution buffers.

Finally, the specificity of each ELISA system was confirmed by testing mixed extracts in non-corresponding assays, e.g. Timothy grass pollen extract assayed with the birch specific ELISA systems.

4. RESULTS AND DISCUSSION

4.1. Allergens

4.1.1. Physico-chemical tests of the cRS

The unformulated cRS were extensively investigated using similar physico-chemical methods [6, 7]. All parameters revealed that the cRS are homogeneous, containing one single fraction of well-folded protein, with no oligomerisation or aggregation detectable. The identity of the purified recombinant proteins was proven unequivocally. The observed MW (17439.6 ± 0.4 Da and 28286 ± 1 Da for Bet v 1 and Phl p 5a respectively) coincided with the theoretical values based on the amino acid sequences.

Physico-chemical characterisation of the formulated and freeze-dried allergen preparations was not possible due to the high albumin content of the samples. The allergen peaks were masked by the HSA peak making identification and/or quantification impossible. It was therefore decided to verify the preservation of the integrity of the allergens and of their biological properties during formulation and freeze-drying by *in vitro* biological tests (see section 4.1.4).

4.1.2. Quantification of protein content by amino acid analysis

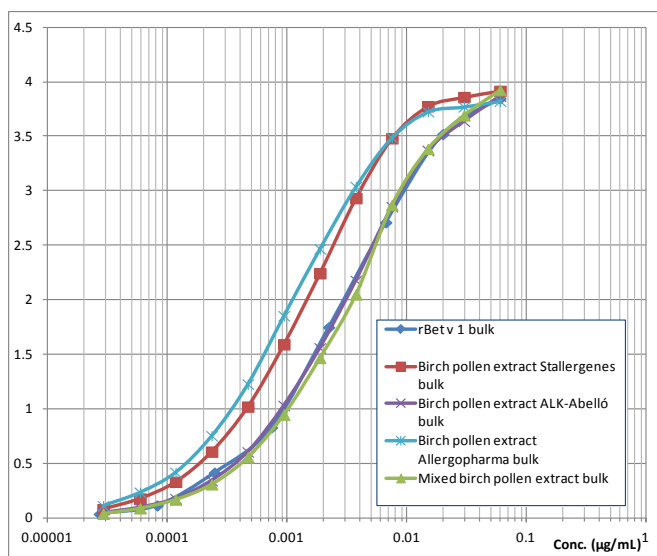
The protein concentrations obtained under GMP conditions by amino acid analysis, i.e. 3.25 ± 0.106 g/L and 1.56 ± 0.026 g/L, for rBet v 1 and rPhl p 5a respectively, were used for the calculation of the protein content after the production of the final batches of the cRS.

4.1.3. Immunochemical tests

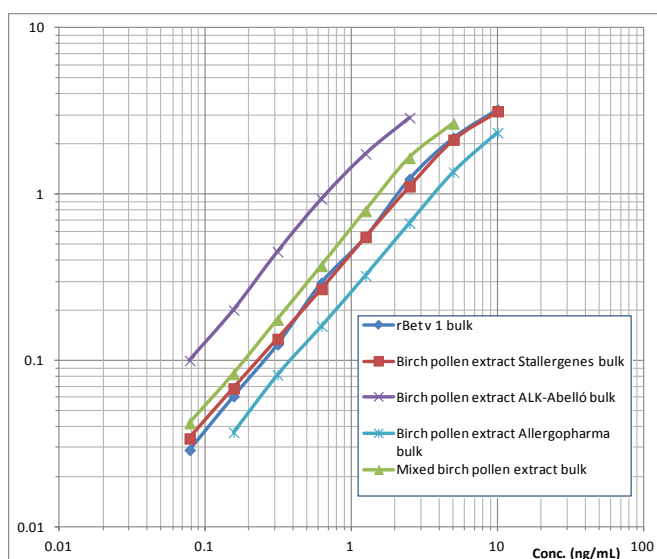
The natural extracts, the cRS (recombinant proteins) and the spiked model samples were tested in the corresponding ELISA systems in order to assess the suitability of the recombinant allergens to be used as reference material for testing natural as well as recombinant commercial products. Bulk material and formulated/lyophilised samples were tested concomitantly to ascertain that the formulation and lyophilisation processes did not lead to degradation or modification of the epitopes, which in turn could alter the recognition of the allergens by the antibodies included in the ELISAs or result in different reaction kinetics.

As shown in Figure 1, in most cases the recombinant allergens reacted similarly compared to the natural allergens in extracts with regard to the slope and OD_{max} of concentration-response curves, in their respective ELISA systems (Table 1). However, this was not the case for the ALK-Abelló Group 5 grass ELISA system. The issue was further investigated and was found to be more likely due to the ELISA system than to the cRS itself. Indeed, a difference in the recognition of the recombinant Phl p 5a and the natural allergen present in the extracts by the capture or detection antibodies might be the reason for the discrepancy observed in the assay. Due to differences in parallelism and OD_{max} (data not shown) the assay could not be included in the subsequent phases of the project.

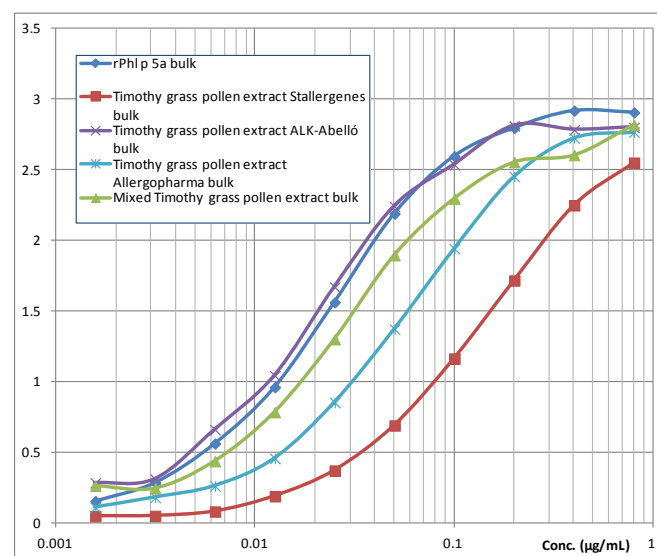
In the remaining ELISA systems both allergens reacted similarly before and after formulation and lyophilisation as shown in Figure 2.



ALK-Abelló Bet v 1 ELISA

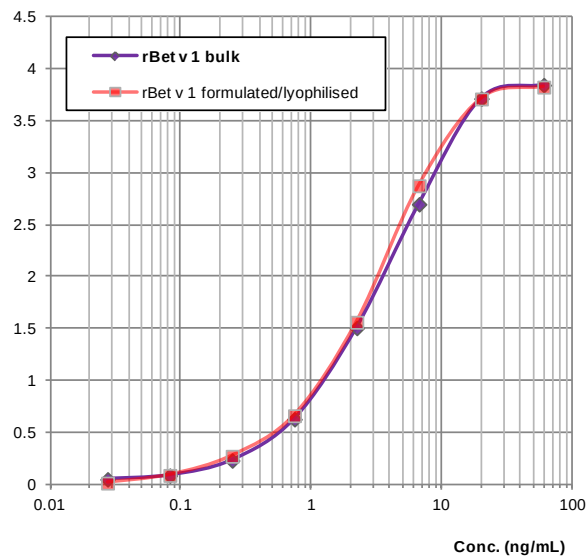


Stallergenes Bet v 1 ELISA

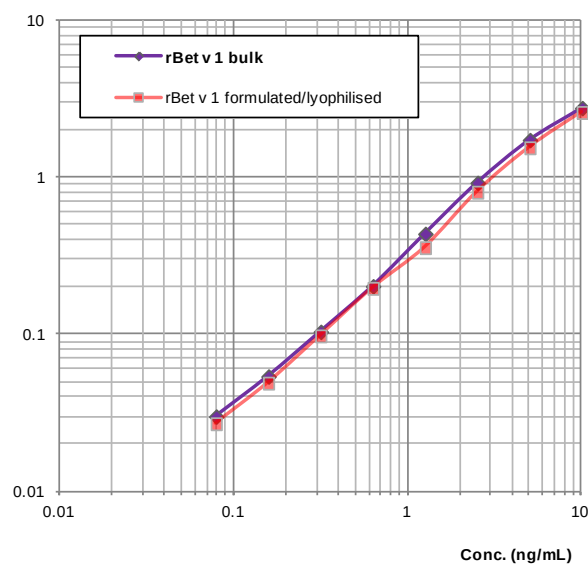


Allergopharma Phl p 5 ELISA

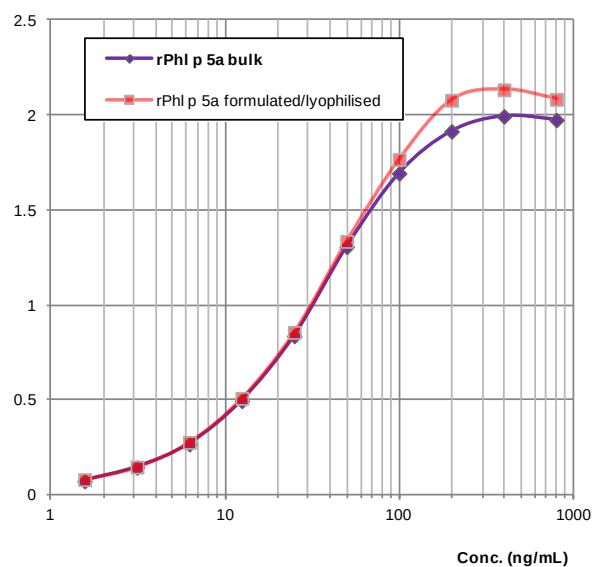
Figure 1 – ELISA systems: comparison of bulk (non-formulated/non-lyophilised) cRS and natural pollen extracts



ALK-Abelló Bet v 1 ELISA



Stallergenes Bet v 1 ELISA



Allergopharma Phl p 5 ELISA

Figure 2 – Parallelism of curves between bulk (non-formulated/non-lyophilised) and formulated/lyophilised CRS

4.1.4. Biological tests

The mean biological responses to the candidate materials were comparable before and after freeze-drying (see Figure 3), i.e. biological activity did not change due to the lyophilisation procedure. The formulated rBet v 1 is even slightly more potent than the non-formulated material. Therefore, the reference materials, the formulation buffer and the freeze-drying procedure were found to be suitable for the next steps of the project.

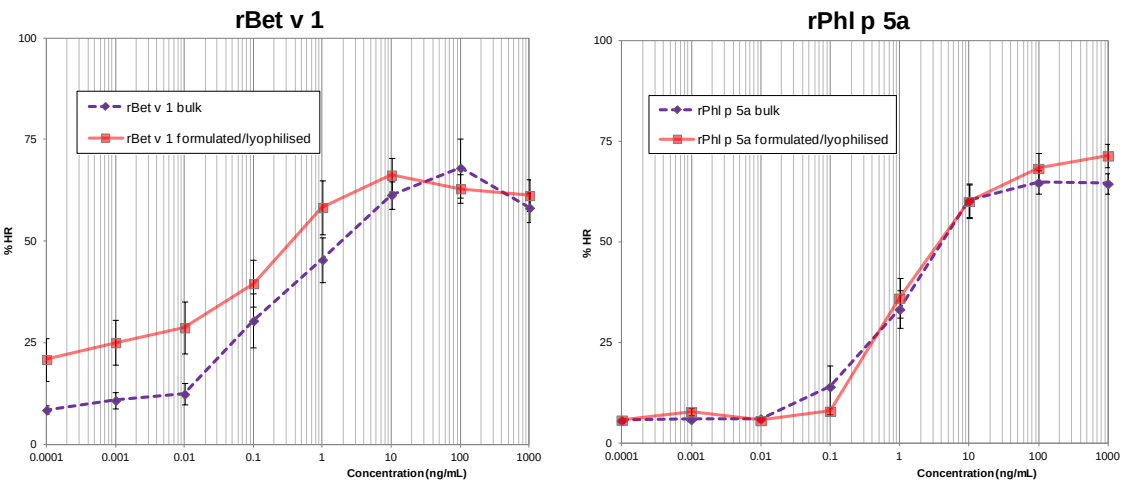


Figure 3 – Comparison of biological activities of bulk (non-formulated/non-lyophilised) and formulated/lyophilised cRS (average of 10 different sera)

4.2. ELISA systems

4.2.1. Reproducibility

The reproducibility and precision of the different ELISA systems were tested on respective bulk and formulated/lyophilised materials. Table 1 shows the parameters (average of *n* independent assays) for each recombinant allergen, before and after formulation and freeze-drying. The reproducibility was found to be appropriate for all ELISA systems.

Table 1 – Parameters of the four-parameter non-linear logistic regression analysis

Bulk cRS				
ELISA system	OD _{min}	Hillslope	ED ₅₀ [ng/mL]	OD _{max}
ALK-Abelló Bet v 1 (<i>n</i> = 17)	0.02 ± 0.06	0.99 ± 0.20	8.29 ± 11.52	4.15 ± 0.22
Stallergenes Bet v 1 (<i>n</i> = 8)	0.02 ± 0.02	1.10 ± 0.16	—	2.08 ± 0.87
Allergopharma Phl p 5a (<i>n</i> = 23)	0.04 ± 0.03	1.35 ± 0.12	49.94 ± 19.22	2.59 ± 0.45
Formulated/lyophilised cRS				
ELISA system	OD _{min}	Hillslope	ED ₅₀ [ng/mL]	OD _{max}
ALK-Abelló Bet v 1 (<i>n</i> = 17)	0.02 ± 0.04	0.99 ± 0.17	4.45 ± 3.08	4.01 ± 0.18
Stallergenes Bet v 1 (<i>n</i> = 8)	0.02 ± 0.01	1.05 ± 0.13	—	2.42 ± 0.71
Allergopharma Phl p 5a (<i>n</i> = 23)	0.06 ± 0.03	1.40 ± 0.14	31.72 ± 3.30	2.07 ± 0.29

4.2.2. Range and limits of detection and quantification

The measurement range spans from the lower to the upper analyte concentrations showing an arbitrarily pre-defined accuracy of 70 to 130% recovery and a mean coefficient of variation (CV) $\leq 15\%$. Therefore, these analyte concentrations represent the Lower and Upper Limits of Quantification (LLOQ and ULOQ) respectively.

From the same set of data, the statistical Limit of Detection (LoD) can be calculated with the following formula:

$$LoD = 3\sigma + x$$

Where σ represents the standard deviation and x the mean value of the negative controls. The calculated ranges and LoDs are given in Table 2.

Table 2 – Summary of measurement ranges and statistical limits of detection

ELISA system	Measurement range (LLOQ–ULOQ) (ng/mL)	LoD (OD)	LoD (ng/mL)
Stallergenes Bet v 1	~ 0.17 to ~ 10	0.262	~ 0.3
ALK-Abelló Bet v 1	~ 0.1 to ~ 6.6	0.078	*
Allergopharma Phl p 5a	~ 2.3 to ~ 130	0.185	~ 0.001

* The value was lower than the lowest analyte concentration. Therefore, it could not be calculated, because an extrapolation of the reference curve was not allowed.

The range, limit of detection and limits of quantification were considered appropriate for all the ELISA systems.

4.2.3. Accuracy and linearity

The mean recovery values were similar between formulated/lyophilised and bulk materials, although slightly higher for the formulated/lyophilised material in the Stallergenes ELISA system, and within the acceptable arbitrary range of 70-130%. The recovery of the spike was calculated by the following formula:

$$Recovery = [(S-U)/T] \times 100$$

Where S is the value obtained for the spiked sample, U the value obtained for the unspiked sample and T the theoretical spiking concentration.

The coefficients of determination (r^2) were between 0.9925 and 0.9994 which indicates a good linear relationship between dose and response. The results are summarised in Table 3. The accuracy and linearity were thus considered to be appropriate for all ELISA systems.

Table 3 – Summary of recovery and linearity values

ELISA System	Overall Recovery (%)		Linearity (coefficient of determination (r^2))	
	Formulated/lyophilised	Bulk	Formulated/lyophilised	Bulk
ALK-Abelló Bet v 1	83.9 $\pm$ 3.7	77.6 $\pm$ 3.1	0.9973	0.9925
Stallergenes Bet v 1	121.9 $\pm$ 2.5	96.0 $\pm$ 1.5	0.9994	0.9992
Allergopharma Phl p 5a	77.4 $\pm$ 11.8	84.1 $\pm$ 4.4	0.9881	0.9915

4.2.4. Robustness

4.2.4.1. Assessment of recovery of the CRS alone

The corresponding CRS were dissolved in a 1:10 diluted NaCl/phenol/glycerol buffer. Six replicates of one appropriate concentration were measured. The mean calculated values and 95% confidence intervals (CI) can be found in Table 4.

Table 4 – Summary of precision and accuracy values of CRS alone

ELISA system	Conc. (ng/mL)	Buffer	Precision				Accuracy			
			Mean	SD	CV	95%-CI	Recovery (%)	95%-CI		
ALK-Abelló Bet v 1	3	Ph/Gly	2.51	0.07	2.93	1.58 3.45	83.7	52.5 114.8		
		ELISA	2.56	0.25	9.95	– 0.68 5.80	85.3	– 22.6 193.2		
Stallergenes Bet v 1	4	Ph/Gly	3.55	0.58	16.22	– 3.76 10.87	88.8	– 94.1 271.7		
		ELISA	3.50	0.31	8.91	– 0.46 7.47	87.6	– 11.5 186.7		
Allergopharma Phl p 5a	30	Ph/Gly	25.42	3.82	15.02	– 23.11 73.95	84.7	– 77.0 246.5		
		ELISA	25.67	2.79	10.87	– 9.79 61.13	85.6	– 32.6 203.8		

Ph/Gly: phenol/glycerol buffer; ELISA: ELISA dilution buffer; SD: standard deviation; CV: coefficient of variation; CI: confidence interval

4.2.4.2. Assessment of recovery in spiked extracts (standard addition method)

The recovery of the spike was calculated by the formula given above. Table 5 shows the mean recoveries and 95% CI. Samples were dissolved in 1:10 and 1:50 diluted NaCl/phenol/glycerol buffer and assayed in 18 replicates over 3 days. One appropriate dilution of the mixed extract spiked with 1 concentration of CRS within the measurement range was measured. Additionally, the mixed extract was assayed without spiking.

Table 5 – Mean recoveries and 95% CI in spiked extracts

		Formulated/lyophilised samples		Bulk samples	
ELISA system	Buffer	Recovery (%)	95%-CI	Recovery (%)	95%-CI
Ph/Gly 1:10					
ALK-Abelló Bet v 1	Ph/Gly	64.7	60.8 – 68.6	62.1	58.7 – 65.6
	ELISA	86.0	80.9 – 91.1	87.2	83.0 – 91.4
Stallergenes Bet v 1	Ph/Gly	91.3	87.5 – 95.0	76.0	71.6 – 80.4
	ELISA	127.2	122.4 – 131.9	100.4	94.0 – 106.7
Allergopharma Phl p 5a	Ph/Gly	60.8	41.9 – 79.7	92.7	74.7 – 110.6
	ELISA	66.8	49.6 – 84.0	72.3	51.4 – 93.3
Ph/Gly 1:50					
ALK-Abelló Bet v 1	Ph/Gly	81.1	74.8 – 87.3	87.9	82.9 – 92.9
	ELISA	87.5	78.4 – 96.6	87.5	80.9 – 94.2
Stallergenes Bet v 1	Ph/Gly	113.0	108.8 – 117.2	93.0	90.5 – 95.6
	ELISA	127.1	123.4 – 130.7	94.0	88.7 – 99.2
Allergopharma Phl p 5a	Ph/Gly	88.0	78.9 – 97.1	110.8	86.4 – 135.3
	ELISA	79.3	70.8 – 87.7	95.3	82.8 – 107.8

Ph/Gly: phenol/glycerol buffer; ELISA: ELISA dilution buffer; CI: confidence interval

The recovery values of samples in 1:10 diluted phenol/glycerol buffer were in general lower compared to those in normal ELISA buffer. Thus it seems that this concentration of phenol/glycerol affects the accuracy of the determination. Moreover, the recovery sometimes does not fall into the accepted range of 70-130%. However, for samples dissolved in 1:50 diluted phenol/glycerol buffer, this effect is no longer observed. In addition, the CV of recovery values was generally low and comparable in both buffer systems for a given sample.

Therefore, all ELISA systems were considered to be acceptable with regard to robustness towards different, sufficiently diluted, dilution matrixes.

4.2.5. Intra- and inter-assay precision

The intra- and inter-assay precision was satisfactory. In all cases, the CV was lower than 15% except for the Phl p 5 assays performed with bulk samples spiked with 5 or 10 ng/mL of recombinant allergen, suggesting a problem with these particular samples.

4.2.6. Specificity

The specificity of each ELISA system was assessed by testing mixed extracts in non-corresponding assays, e.g. Timothy grass pollen extract assayed with the birch specific ELISA systems. No evidence of cross-reactivity was observed with any of the ELISA systems used.

5. CANDIDATE REFERENCE STANDARDS

Taking account of the protein concentration determined by amino acid analysis under GMP conditions (see section 4.1.2), the nominal content of the vials is 10.25 µg/vial and 8.56 µg/vial for rBet v 1 and rPhl p 5a respectively. Around 3000 vials of each CRS were produced by filling 1 mL of formulated recombinant allergen preparation into 3 mL white glass vials using a validated procedure. Appropriate controls were performed to check the homogeneity of the batch as well as the residual water content of the lyophilised materials. The mean filling weight was assessed over 10 vials specifically labelled and placed at the beginning, middle and end of the fill and was 0.9956 ± 0.0022 and 0.9964 ± 0.0015 g/vial for rBet v 1 and rPhl p 5a respectively. Twelve additional vials were specifically labelled as in-process controls and placed at the beginning, middle and end of the filling process and were freeze-dried together with the rest of the batch. These vials were subsequently checked for the homogeneity of the fill. The vials from beginning, middle and end of the fill showed identical activity in the ELISA. Therefore, the fill was considered to be homogenous. In addition, the activity of the lyophilised control vials did not differ from the non-lyophilised/formulated control vials.

The residual water content in the lyophilisate was measured on 8 vials taken at random from each batch. For both allergens, the residual water content was found suitable with long term storage i.e. 1.5% and 1.6% for rBet v 1 and rPhl p 5a respectively.

Thus all controls show that the CRS are suitable for the intended purpose. The CRS will be stored at – 20 °C.

6. MONITORING

A subset of 30 vials for each CRS was set aside at – 80 °C as reference for real-time stability monitoring. To avoid degradation due to hydration of the samples in case of deterioration of the screw caps, the vials were placed into aluminium bags containing a sufficient number of containers for one monitoring assay, i.e. 3 vials, and an appropriate water-adsorbing agent according to the EDQM internal SOP. The CRS stored at – 20 °C will be monitored at regular intervals against the material stored at – 80 °C, using appropriate ELISAs.

7. SUMMARY AND CONCLUSIONS OF THE FEASIBILITY TESTING (PHASE 1)

7.1. Allergen reference standards

During the pre-qualification phase (Phase 1) of the BSP090 project, the recombinant purified allergen preparations rBet v 1 from Stallergenes and rPhl p 5a from Allergopharma were submitted to extensive tests and found to be suitable as cRS. The formulation buffer and lyophilisation process taken from the CREATE project were adopted as such (buffer) or adapted (lyophilisation) and their suitability was checked. The validated procedure was then applied to the production of the cRS. The formulated/lyophilised cRS were then tested to ensure that their activity was similar to that of natural allergen extracts and to non-formulated, non-lyophilised material. In addition, the control samples showed that the cRS are homogeneous over the whole production process and the residual water content is appropriate for long term storage.

Thus, both formulated/lyophilised recombinant allergen preparations were found to be suitable as cRS and subsequently included in the extended feasibility phase of the project (Phase 2).

7.2. ELISA systems

From the four ELISA systems tested during the pre-qualification phase, only three were found suitable to be included in the extended feasibility phase (Phase 2) for further assessment. Indeed, the ALK-Abelló Group 5 grass ELISA showed some discrepancies in the recognition of the recombinant allergen when compared to the natural counterpart. It could therefore not be further considered as a reference method for the quality control of all Group 5 grass pollen allergen preparations and was thus dropped. Since all preparations of recombinant allergens showed identical activities in the three remaining ELISA systems, all of them were included in Phase 2.

8. EXTENDED FEASIBILITY STUDY (PHASE 2)

8.1. Aim of the study

The aim of the extended feasibility study was to investigate whether the ELISA systems could be transferred to other laboratories and, if possible, select one Bet v 1 ELISA system for the international collaborative study (Phase 3). If both Bet v 1 ELISA systems were to perform equally, they would both be included in the next phase. In addition, the suitability of the rBet v 1 and rPhl p 5a preparations as RS was further investigated.

8.2. Materials, methods and statistical analysis

Three Official Medicines Control Laboratories (OMCLs) participated in this phase of the study. They are listed in section 10. Laboratories were coded for the study and the order of listing does not necessarily correspond to the numerical codes.

Model samples were prepared by spiking natural extracts with their respective recombinant allergen (see section 3.1.3). These model samples were tested in parallel in all 3 ELISA systems in the 3 different laboratories to investigate transfer of the methods and applicability by different operators.

In addition, formulated and freeze-dried cRS and run controls were provided to the laboratories. The same vial of reconstituted candidate rBet v 1 was used for both Bet v 1 ELISAs to avoid variations due to the reconstitution.

As for Phase 1, assay specific ELISA reagents such as pre-coated microtitre plates (MTPs) or antibodies were provided to the EDQM by the allergen manufacturing companies and

dispatched centrally to the participants. An overview of the ELISA reagents used for the extended feasibility phase is given in Table 6. Detailed information on the reconstitution and/or dilution of ELISA reagents was given in the study protocol. Additional commercially available reagents for the preparation of buffers and working solutions were purchased by the participants following recommendations (i.e. producers, purity) from the manufacturers.

Table 6 – ELISA reagents used in the extended feasibility phase

Sample	EDQM internal number	Manufacturer batch number	Concentration/content	Storage/shipment
ALK-Abelló				
MTPs coated with anti-Bet v 1 MAb	35817	3/08	10 plates	+ 4 °C
Anti-Bet v 1 rabbit serum	35815	1/02	2 × 30 µL	– 80 °C
Goat anti-rabbit IgG-POD	35814	D35447	2 × 30 µL	+ 4 °C
Allergopharma				
MAbs 1D11C8	35783	VO 72/42	2 × 5 aliquots (1 aliq. = quantity for 1 plate)	– 20 °C
MAbs BO1 biot.	35784	VO 77/14	2 × 5 aliquots (1 aliq. = quantity for 1 plate)	– 20 °C
MTPs Greiner	35785	65061	10 plates	RT
Streptavidin-POD	35786	S-5512	2 × 2 mg	– 20 °C
ABTS tablets (10 mg)	35787	A-9941	2 × 5 tablets	– 20 °C
Stallergenes				
Capture Abs 5B4	35813	E7053	2 × 125 µL (2.964 g/L)	– 20 °C
Detection Abs 6H4 biot.	35812	E6110	2 × 30 µL	+ 4 °C

RT: room temperature

Participants were requested to test the model samples in duplicate with the corresponding ELISA systems, as described in the respective protocols, in 3 independent assays, on three different days, by one operator and to strictly comply with the instructions given in the protocols.

The participants reported their results following the calculation method described by the manufacturers. The statistical analysis of all data was performed at the PEI and the EDQM by independent methods to investigate the influence of different calculation methods on the results. The statistical analysis at the PEI was done by means of the parallel line assay method. For the calculations performed at the EDQM, the model used was a 4-parametric logistic curve for all ELISA systems, except for the results from Laboratory 3 with the Stallergenes ELISA system, where a 3-parametric exponential curve was used. The values obtained by these approaches were compared to each other and also to those calculated by the participants.

The allergen concentrations were calculated relative to the respective cRS, with 95%-confidence limits, for the unspiked and spiked extracts, and compared with the initial measurements from PEI (hereafter named PEI specification) in order to specify the recovery

of each sample. Moreover, the recovery of the spiked concentration in extract (extract B) and buffer (controls) was calculated relative to the theoretically spiked concentration. The measured spike in extract B was calculated from the estimated concentrations from extracts A and B (measured spike = extract B – extract A (unspiked)). The mean recoveries for all 3 days were calculated by combining the individual assay results. The measurement uncertainty, the inter-laboratory precision and repeatability expressed as coefficients of variation, estimated by means of a mixed linear model for each allergen and extract with random factor laboratory, are summarised in Table 7.

8.3. Results

The overall uncertainty was generally low ($CV \leq 15\%$) and comparable for both extracts except for extract A in the Allergopharma ELISA system ($CV 25\%$). For all systems, a good recovery was observed for both extracts with overall confidence intervals within the predefined empirical specifications of 70-130%.

The difference between calculations performed by the statisticians (PEI and EDQM) and the laboratories is in general small when the regression curves are good but different calculation schemes can occasionally lead to non-negligible differences although this is usually only the case when the assay should be declared invalid or when data is of unsatisfactory quality. It may therefore be necessary to set strict validity criteria for each assay.

Table 7 – Summary of calculated precision parameters (CV%)

		Extract A	Extract B	Control 1	Control 2
ALK-Abelló	Overall measurement uncertainty	13.50	12.30		
	Inter-lab precision	10.80	n.e.	28.40	34.40
	Repeatability	8.00	12.30		
Stallergenes	Overall measurement uncertainty	15.80	12.80		
	Inter-lab precision	9.80	n.e.	8.60	11.60
	Repeatability	12.30	12.80		
Allergopharma	Overall measurement uncertainty	25.00	11.60		
	Inter-lab precision	19.80	n.e.	9.40	13.00
	Repeatability	15.30	11.60		

Extract A: unspiked; Extract B: spiked with the respective CRS at a given concentration; Control 1, control 2: run controls of the respective CRS at a known concentration; n.e.: not estimated

8.4. Discussion

Problems have been encountered during the experiments for the establishment of the specifications at the PEI, except for the ALK-Abelló ELISA system. Therefore, it was necessary to evaluate the recovery with regard to the theoretical spike value and not solely versus the measured spike concentration.

Nevertheless, for both Bet v 1 ELISAs a good recovery relative to the specifications was observed with a slight tendency for underestimation for both extracts. However, the overall

CI never exceeded the pre-defined empirical limits of 70-130% thus both assays can be considered suitable for the detection of Bet v 1 in birch pollen extracts. However, the ALK-Abelló ELISA system seems to be more appropriate for the quantification of the total amount of Bet v 1 in extracts whereas the Stallergenes ELISA system seems to have a better capacity for the detection of small differences in Bet v 1 content. This might be due to a different sensitivity of the ELISA systems.

With regard to the Phl p 5 ELISA system from Allergopharma, for the unspiked extract A a good recovery was achieved by Laboratories 1 and 2 only, with a tendency for a slight overestimation (mean recovery 112%). A clear overestimation was observed (mean recovery 158%) for Laboratory 3 but this was estimated to be an experimental problem rather than due to the ELISA system itself. Extract B showed for all participants consistently 20% more content than expected from the specifications and the overall CI exceeded slightly the allowed defined constraints (70-130%). However, relative to the theoretical spike concentration, the overestimation was not observed (mean recovery 104%).

Precision of individual as well as overall results could be regarded as acceptable for this kind of analysis. The measurement uncertainty was equal or below 15% (with the exception of extract A in the Allergopharma system). Inter-laboratory precision was, due to the limited amount of data, only estimable with this model for extract A. The CVs, ranking from 9.8% for Stallergenes, 10.8% for ALK-Abelló to 19.8% for Allergopharma, indicate a relatively good consistency of content estimation among the participating laboratories. Repeatability (precision between assay replications i.e. different days) was also acceptable with CV values below 16%.

8.5. Conclusion on Phase 2

Both candidate materials were thoroughly investigated in Phases 1 and 2 of the project and were found to be suitable as cRS.

The Phl p 5 ELISA system from Allergopharma performed well in both Laboratories 1 and 2. The slight overestimation observed relative to specifications established at the PEI was not confirmed when compared to the theoretical value of the spike. This might be a hint that an experimental error occurred during the measurement of the specification value of extract B after manufacturing of the model samples at PEI. The overall inter-laboratory precision, repeatability and measurement uncertainty were very good. In conclusion, the Phl p 5 ELISA system from Allergopharma was considered suitable to be further explored in the next phase of the study.

Both Bet v 1 ELISA assays were comparable with regard to their performance in the 3 participating laboratories. Thus, it was decided to include both assays in the collaborative study (Phase 3).

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10. PARTICIPANTS OF PHASE 2 (in alphabetical order by country)

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11. ABBREVIATIONS

BSP: Biological Standardisation Programme; CI: Confidence interval; CREATE: Development of Certified Reference Materials for Allergenic Products and Validation of Methods for their Quantification; CRS: candidate reference standards; EDQM: European Directorate for the Quality of Medicines & HealthCare; ELISA: Enzyme Linked Immuno-Sorbent Assay; EU: European Union; GMP: Good Manufacturing Practices; HSA: Human serum albumin; IgE: Immunoglobulin E; IHR: In-house Reference; IL: Interleukin; IUIS: International Union of Immunological Societies; kDa: Kilodaltons; LLoQ: lower limit of quantification; LoD: limit of detection; LoQ: limits of quantification; MAb: monoclonal antibody; MTP: Microtitre plate; MW: Molecular weight; OD: Optical Density; OMCL: Official Medicines Control Laboratory; PEI: Paul-Ehrlich Institut; Ph. Eur.: European Pharmacopoeia; POD: peroxidase; r: recombinant; RS: reference standard; SOP: Standard Operating Procedure; ULoQ: upper limit of quantification.

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