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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC MOLECULAR WEIGHT DETERMINATION OF ALLERGEN EXTRACTS

EXAMINATION OF THE INFLUENCE OF THE COLUMN MATERIAL ON ALLERGENIC ACTIVITY AND ALLERGEN PATTERNS

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SUMMARY

To investigate whether the column material employed in size-exclusion high-performance liquid chromatography (HPLC) influences the allergenic activity and antigen/allergen patterns of allergen extracts, the molecular weights of a mite and a birch pollen allergen extract were determined using a Bio-Sil TSK 250 column with a guard column. The allergenic acitivities were measured by RAST inhibition and the antigen/allergen patterns were determined by crossed (radio)immunoelectrophoresis. The original extracts and the corresponding eluates after HPLC runs showed the same allergenic activity and the same number of antigen/allergen precipitation lines. Only slight differences in the peak heights of some precipitates were observed.

INTRODUCTION

The active components in allergen extracts are proteins and glycoproteins with different molecular weights, so the determination of their molecular weights is an important analytical criterion, especially for in-process control of production. Traditionally, molecular weight determinations of allergen extracts were carried out by gel filtration[1] or sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis[2]. However, both of these methods are time consuming. Moreover, using the SDS method only subunits of the proteins are analysed. For these reasons, a rapid and precise method for the determination of the molecular weights of the native proteins would be useful. Florvaag *et al.*[3] and Maasch *et al.*[4] used size-exclusion high-performance liquid chromatography (HPLC) for molecular weight determinations in native and modified allergen preparations, and reduced the time necessary for the analysis from *ca.* 10 h using gel filtration to 35 min.

The application of HPLC to the preparative separation of fractions from allergen extracts was reported recently by Calam *et al.*[5] and Haavik *et al.*[6]. The successful application of HPLC to analytical and preparative work in allergen extract

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research requires an inert separation material in the column, ensuring complete recovery of the allergenic activity in the eluate.

In this work we investigated whether the column material used for size-exclusion HPLC influences the allergenic activity and the antigen/allergen patterns of dialysed birch pollen and mite allergen extracts. Finally, we tested the reproducibility of HPLC for the molecular weight determination of allergen extracts by twelve independent runs of the same allergen preparations.

EXPERIMENTAL

Allergen extract preparations

We prepared allergen extracts from pollens of birch (*Betula verrucosa*) (Allergon, Engelholm, Sweden) and purified mite bodies of *Dermatophagoides pteronyssinus* (Commonwealth Serum Laboratories, Melbourne, Australia). Extractions were carried out at 4°C with Coca's solution without phenol overnight. Subsequently, the extracts were dialysed[7] and lyophilized.

Prior to separation, the lyophilized allergen extracts were reconstituted to a protein concentration of 7.5 mg/ml[8]. For each run, 20 μl of these solutions were injected. Eluates from five injections were collected and blended (*ca.* 150 ml). In parallel, an equal amount of protein of the original lyophilized allergen extracts was dissolved in 150 ml of 0.5 *M* ammonium acetate buffer (pH 6.5). For further specific investigations, these two samples were desalted using Visking dialysis tubing (Serva, Heidelberg, F.R.G.) with a cut-off of 8000–15 000 daltons. Subsequently, the extracts were lyophilized and dissolved in equal volumes of doubly distilled water.

HPLC system

The HPLC system employed consisted of a 300 × 7.5 mm I.D. Bio-Sil TSK 250 column (separation range 1000–300 000 D) with a guard column (Bio-Rad Labs., Munich, F.R.G.), a constametric pump, a Rheodyne 7125 injection valve, a Model D UV monitor (254 nm) and a type 301 integrator (Milton Roy, Hasselroth, F.R.G.). As the solvent, 0.5 *M* ammonium acetate buffer (pH 6.5) (Merck, Darmstadt, F.R.G.) was used at a flow-rate of 0.9 ml/min. The system was calibrated with a Bio-Rad Labs. calibration kit, containing a mixture of proteins of 1350–670 000 daltons.

Measurement of allergenic activity using RAST inhibition

To establish whether the allergenic activity of the extracts is influenced by the column material used for HPLC, the allergenic activity of the desalted original extracts and of the corresponding eluates were determined by RAST inhibition[9]. Briefly, different dilutions of allergen extracts were incubated in test-tubes (55 × 12 mm) with dilutions of pooled sera from patients allergic to birch pollen or mites. These mixtures were incubated overnight at room temperature with allergen-coupled paper disks[10]. After washing steps, rabbit ^{125}I-labelled anti-human IgE (Fc specific) (Pharmacia, Freiburg, F.R.G.) was added as tracer for IgE. After a second overnight incubation, the allergen disks were washed carefully and counted for bound radioactivity in a gamma counter. The allergenic activity of an extract was determined by its capacity to induce 50% inhibition of uptake of allergen-specific IgE by the allergen disks in comparison with an uninhibited sample.

Measurement of antigens and allergens by crossed (radio)immunoelectrophoresis (CIE/CRIE)

The patterns of antigens and allergens of the desalted original extracts and the corresponding eluates were determined by CIE/CRIE. CIE was performed as described by Løwenstein[11], employing 28 μl of each sample. Volumes of 50 μl of the rabbit antisera (ALK, Copenhagen, Denmark) per millilitre of separation gel were used, *i.e.*, anti-*Dermatophagoides pteronyssinus* and anti-*Betula verrucosa*.

Separation in the first dimension was carried out for 30 min at 10 V/cm and 15°C and in the second dimension for 17 h at 2 V/cm. CIE plates were washed with 0.1 *M* sodium chloride solution and in distilled water and finally dried in a stream of cold air. For CRIE, the method of Weeke and Løwenstein[12] was used with the following modifications: each CIE plate was incubated with 12 ml of a diluted serum pool (1:24) collected from patients with known sensitivity to birch or mites. As a tracer for bound IgE we used *ca.* 400 nCi of ^{125}I-labelled anti-IgE (Pharmacia) in 12 ml of buffer. Each incubation was carried out overnight with gentle horizontal shaking. From each CRIE plate three autoradiographs were produced by exposing Kodak X-Omat AR films at −20°C for 3, 7 and 14 days. The CIE plates were finally stained with Coomassie Brillant Blue R 250.

Measurement of reproducibility of HPLC

To assess the reproducibility of size-exclusion analysis by HPLC, dialysed birch pollen and mite allergen extracts were analysed in twelve independent runs. The retention times and percentage areas of the prominent peaks in each measurement were compared.

RESULTS

HPLC of allergen extracts and assessment of reproducibility

Fig. 1 shows the HPLC traces of the dialysed birch pollen and mite allergen extracts. The mean retention times and the mean percentage areas of the prominent

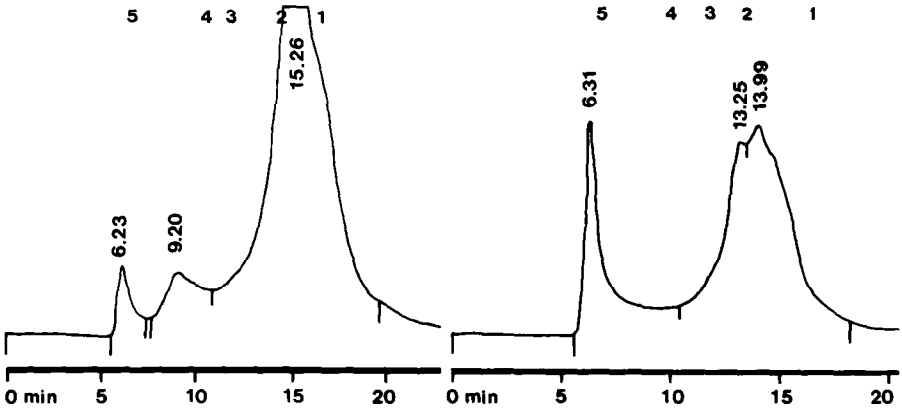

Fig. 1. HPLC molecular weight determination of mite and birch pollen allergen extracts with a Bio-Sil TSK 250 column with guard column. Solvent: 0.5 *M* ammonium acetate (pH 6.5), flow-rate 0.9 ml/min. Left, chromatogram of the dialysed mite allergen extract; right, chromatogram of the dialysed birch pollen allergen extract. 1–5 indicate RTs of the calibration substances: (1) vitamin B_{12} (1350 daltons); (2) myoglobin (horse) (17 000 daltons); (3) ovalbumin (chicken) (44 000 daltons); (4) gamma-globulin (bovine) (158 000 daltons); (5) thyroglobulin (bovine) (670 000 daltons).

TABLE I

MEAN RETENTION TIMES AND PERCENTAGE AREAS OF THE PROMINENT PEAKS OF DIALYSED BIRCH POLLEN AND MITE ALLERGEN EXTRACT (n = 12) AND RESULTS OF MOLECULAR WEIGHT DETERMINATIONS

Allergen extract	t_R *(min)*	*C.V. (%)*	*MW (D)*	*Peak (%)*	*C.V. (%)*
Birch pollen	6.31	0.25	>670 000	27.64	2.40
Birch pollen	13.23	0.30	14 125	26.35	5.17
Birch Pollen	13.95	0.19	7940	45.91	2.65
Mite	6.28	0.58	>670 000	2.27	15.00
Mite	9.21	0.33	281 000	6.34	2.16
Mite	15.28	0.14	5011	90.33	1.29

peaks are shown in Table I. The molecular weights were determined by using the regression line of a calibration graph (Fig. 2) obtained with a calibration kit. The coefficients of variation (C.V.) of retention times calculated from twelve independent runs of the dialysed birch pollen and mite allergen extracts were in the range 0.14–0.58% and for the percentage peak areas in the range 1.29–15.0%, indicating good reproducibility of the analysis.

Measurement of allergenic activity using RAST inhibition

The allergenic activities of the samples were determined by RAST inhibition. The regression lines of the RAST inhibition curves for the original samples and those of the corresponding eluates were nearly congruent (Fig. 3). Using a statistical programme[13] we were unable to find significant differences in the 50% inhibition values and slopes of the curves. The relative potency (P_{rel}), calculated from the quotient of the 50% inhibition values of reference (original sample) and sample (corresponding eluates), was 1.08 for birch pollen allergen extracts and 0.97 for mite allergen extracts.

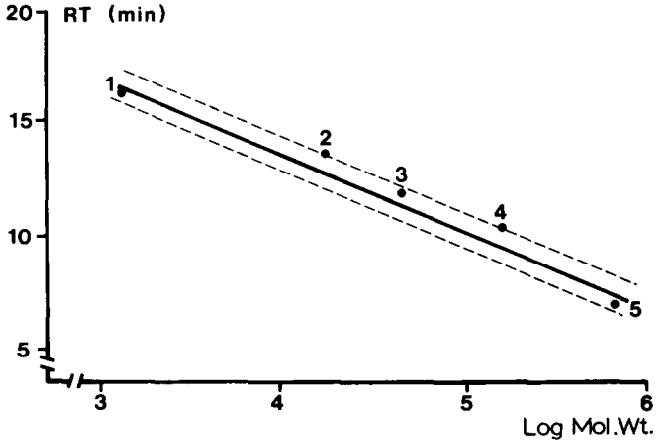

Fig. 2. Regression line of the calibration graph of a mixture of proteins: (1) 1350 daltons; (2) 17 000 daltons; (3) 44 000 daltons; (4) 158 000 daltons; (5) 670 000 daltons; $p < 0.01$. ----, Confidence limit.

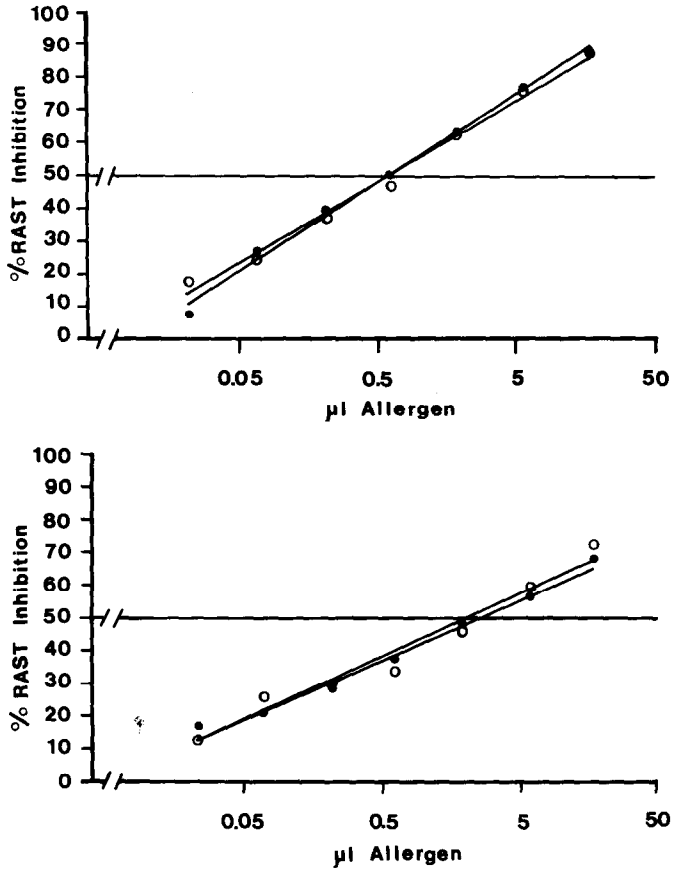

Fig. 3. Regression lines of the RAST inhibition of the original samples and corresponding eluates of mite and birch pollen allergen extracts. Top, RAST inhibition of the mite allergen extracts; bottom, RAST inhibition of the birch pollen allergen extracts. ●, Original samples; ○, corresponding eluates.

This indicates equal allergenic activities and compositions of the allergenic determinants of the samples before and after separation by HPLC.

Determination of the number of antigens and allergens by CIE/CRIE

The antigen/allergen patterns of the samples were determined by CIE/CRIE. As shown in Fig. 4 and Table II, birch pollen and mite allergen extracts showed nearly the same CIE/CRIE patterns and the same number of antigens (precipitation lines) and allergens (allergen bands) in the original samples and the corresponding eluates when analysed on an equal-volume basis.

DISCUSSION

Molecular weight determination is often used as a descriptive analytical method for allergen extracts. Especially for modified allergen preparations, molecular weight determination is part of the final quality control[4]. The method most commonly employed for this purpose is gel filtration[1]. However, this method is very time

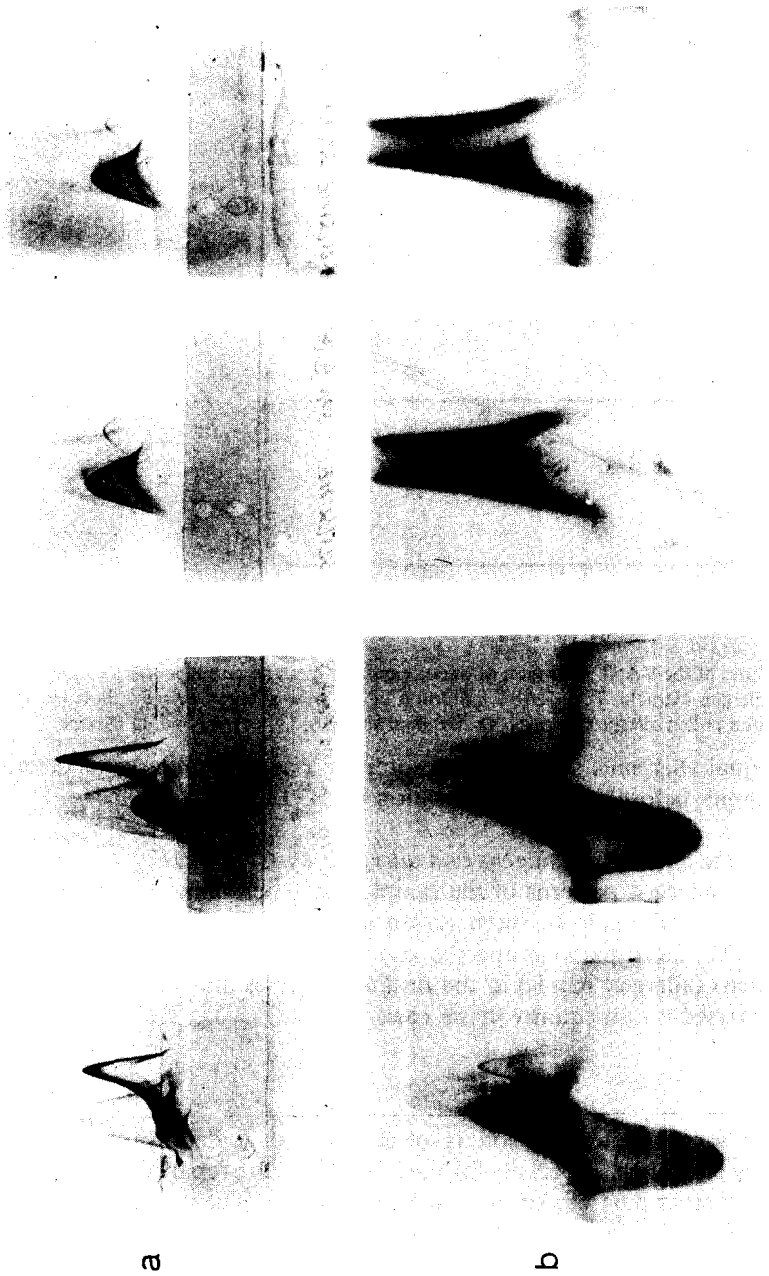

Fig. 4. CIE/CRIE of the desalted allergen extracts. (a) CIE; (b) CRIE. Left-hand pairs, original sample of mite allergen extract (M_1) and corresponding eluate (M_2); right-hand pairs, original sample of birch pollen allergen extract (B_1) and corresponding eluate (B_2).

TABLE II

NUMBER OF ANTIGENS [PRECIPITATION LINES (PL)] AND ALLERGENS [ALLERGEN BANDS (AB)] OF THE ORIGINAL SAMPLES OF MITE AND BIRCH POLLEN ALLERGEN EXTRACTS AND OF THE CORRESPONDING ELUATES DETERMINED BY CIE/CRIE

Allergen extract	*PL*	*AB*
Mite, original sample	8	7
Mite, corresponding eluate	8	7
Birch pollen, original sample	8	4
Birch pollen, corresponding eluate	8	4

consuming and particularly when molecular weight determinations of allergen extracts need to be carried out routinely, *e.g.*, for in-process control of production, an extended analysis time causes problems. The application of HPLC for this purpose were reported recently[3,4], and the time necessary for molecular weight determination could be reduced by a factor of almost 1:20, *i.e.*, from *ca.* 10 h by gel filtration to 35 min. Hence molecular weight determinations of allergen extracts by HPLC seems suitable for the purposes of in-process control, the testing of the consistency of different batches of allergen extracts and the analysis of chemically modified allergen extracts.

When using HPLC for molecular weight determinations of allergen extracts, it is essential that the activity of the samples is not retained or influenced by the column material employed, especially if HPLC is to be used in preparative work.

By RAST inhibition measurements on the original samples and the corresponding eluates we were able to show that the allergenic activity of the extracts was not influenced by the column material. The original extracts and the corresponding eluates were of the same allergenic activity. This was confirmed by CIE/CRIE. The original samples and the corresponding eluates showed the same number of antigen/allergen bands and nearly the same antigen/allergen patterns. The slight differences in the peak heights of the antigen/allergen precipitates in CIE/CRIE of the original mite allergen extracts in comparison with that of the corresponding eluates must be attributed to the variability of the method.

If HPLC is to be applied as an analytical tool for in-process control and monitoring the consistency for different production batches of allergen extracts, high reproducibility of the method is essential. As can be seen in Table I, HPLC molecular weight determinations of allergen extracts showed good reproducibility. In twelve independent measurements of birch pollen and mite allergen extracts, the coefficients of variation of the retention times of the prominent peaks of the birch pollen and mite allergen extracts were in the range 0.14–0.58% and for the percentage peak areas in the range 1.29–15.0%.

According to the recommendations of the manufacturer (Bio-Rad Labs.), the molecular weight range of the Bio-Sil TSK 250 column is 1000–300 000 daltons. The regression line of the calibration graph (protein mixture of 1350–670 000 daltons) (Fig. 2) showed a highly significant correlation between molecular weight and retention time ($p < 0.01$), *i.e.*, molecular weight determinations with the TSK 250 column can be carried out in the range 1350–670 000 daltons.

The column material employed was extremely stable. By using clear solutions, a well degassed solvent and a guard column, the lifetime of the Bio-Sil TSK 250 column could be maintained over a long period. Only after 1500 injections did the guard column show the first signs of damage. We were able to continue to work with the same Bio-Sil TSK 250 column after changing the guard column.

In conclusion, these studies show that the column material used for HPLC does not influence the allergenic activity and the antigen/allergen patterns of the extracts. Hence it is possible to isolate components of allergen extracts by HPLC prior to further investigation by more specific biochemical and immunological methods.

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