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ISOTACHOPHORESIS OF ALLERGENIC EXTRACTS

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SUMMARY

One aspect of the isotachophoretic determination of protein patterns in biological samples of interest is the characterization of allergens. This group of (glyco) proteins, causing allergic reactions, is used both for diagnosis and in the treatment of allergy. The aim of this investigation was to obtain a maximum amount of information, within one run, on the (glyco)protein composition of a number of allergenic extracts (*e.g.*, from pollen or house dust mites). Commercially available extracts were dialysed prior to analysis to remove disturbing buffer constituents. A high-pH system was chosen in order to obtain a maximum amount of information from the samples (1–2 μl). The leading electrolyte was 0.01 *M* Cl^-, buffered with Tris (pH 8.2), containing 0.2% w/v hydroxyethylcellulose, and the terminating electrolyte was β-alanine, buffered to pH 10 with $Ba(OH)_2$. The total analysis time was 15–20 min using a PTFE capillary (0.2 mm I.D.). The pre-separation current was 30 μA and the current during detection was 15 μA. UV absorption was measured at 280 nm. For optimal discrimination of the compounds of interest, an ampholyte mixture was used for spacing. The analytical procedure yielded highly reproducible UV patterns. Significant differences between various allergenic extracts were observed.

It was concluded that isotachophoresis is a powerful method for the physico-chemical characterization of individual allergenic extracts, *e.g.*, with respect to manufacturing and quality control.

INTRODUCTION

Allergens are predominantly proteins or glycoproteins and induce an allergic reaction in certain individuals after contact with the airways, mucous membranes, skin or gastrointestinal tract. A large number of allergenic molecules are known and they can be derived from many sources, including pollen, house-dust mites, fungi, animal epithelia, insects, bacteria, foods and drugs. Each of these sources contains more than one allergen[1,2].

In industrialized countries about one in every six individuals will develop an

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allergic reaction after contact with an allergen. Both for diagnosis and treatment of allergy, extracts from the material causing that allergy are used. These so-called allergenic extracts should be characterized as carefully as possible in order to compare and quantify their diagnostic value and therapeutic efficacy[3]. International cooperative efforts have provided substantial progress in the field of regulatory control and standardization[4–6]. In fact, allergenic extracts are poorly defined mixtures of substances and a number of methods have therefore been worked out to analyse both the chemical composition and biological activity of such extracts.

For chemical consistency analysis, isoelectric focusing has been widely used for the identification of allergens[7]. Other electrophoretic techniques include crossed immunoelectrophoresis[8], crossed radioimmunoelectrophoresis[9] and isotachophoresis[10]. Chromatographic procedures have also been employed, *e.g.*, gel filtration and exchange procedures[11] and high-performance liquid chromatography[12]. Recently, polyacrylamide gel electrophoresis, followed by protein blotting on to nitrocellulose paper, has been added to the methods for the characterization of allergenic extracts[13].

In this study we used an alternative electrophoretic procedure for the protein profiling of allergenic extracts, *viz.*, isotachophoresis (ITP)[14]. In comparison with all of the other techniques mentioned above, the analysis time in ITP is short (15–20 min) and the analytical data for a specific sample are directly available. ITP analysis of pollen allergens has been described previously in a short paper[10]. The aim of this investigation was to obtain further information as to whether extracts from a number of different allergenic sources show characteristic electrophoretic profiles. Further, the reproducibility of such a "fingerprint" analysis and the effect of storage under practitioners' conditions were determined.

EXPERIMENTAL

Equipment

The isotachophoretic experiments were carried out using equipment developed and built by Everaerts *et al.*[14]. This equipment differs from that commercially available (LKB or Shimadzu) in that the separation capillary is *ca.* 0.2 mm instead of *ca.* 0.5 mm I.D., which results in a higher resolution and less pronounced radial temperature and zone-boundary profiles. A higher current density, therefore, enables a shorter analysis time to be achieved.

Operational system

The operational system used (Table I) is a high-pH anionic system, which means that in preparation and during analysis precautions have to be taken in order to avoid interference from the carbonate originating from the chemicals used and from air. These problems and their solutions have been considered earlier[15,16]. It was found convenient to prepare a stock solution of 0.2% hydroxyethylcellulose (HEC), which was treated with a Type V mixed-bed ion exchanger (Merck, Darmstadt, F.R.G.). This was stored in a refrigerator after addition of HCl to a concentration of 0.01 *N*.

In a 25-ml disposable plastic syringe, 46 mg of trishydroxymethylaminomethane (Tris) was dissolved in 20 ml of the above-mentioned HEC–HCl solution. In this way contact with air is minimized. For each series of runs a fresh solution must be prepared.

TABLE I

OPERATIONAL SYSTEM FOR PROTEIN PROFILING OF ALLERGENIC EXTRACTS

Separation at 30 μA in a 250 × 0.2 mm I.D. capillary.

Parameter	*Leading electrolyte*	*Terminating electrolyte*
Anion	Chloride	β-Alanine
Concentration	0.01 *M*	*ca.* 0.01 *M*
pH	8.0	9–10
Counter ion	Tris	$Ba(OH)_2$
Additive	0.2% HEC	–

For the terminator, *ca.* 20 mg of β-alanine and *ca.* 60 mg of $Ba(OH)_2$ were dissolved in 20 ml of water in a similar manner. The $Ba(OH)_2$ was used to increase the pH and to precipitate carbonate. To prevent the precipitate from entering the isotachophoretic equipment, the terminator syringe was connected with a 0.45-μm Luer-lock type disposable filter (Millipore, Bedford, MA, U.S.A.). The terminating electrolyte solution should be freshly prepared for each series of runs.

Electrophoretic conditions

The separation was carried out at 30 μA, during which the voltage increased from 2 to 10 kV. Then, before detection, the current was reduced to 15 μA. The end voltage was approximately 15 kV with a total analysis time of *ca.* 15 min. Detection was carried out by UV absorption at 280 nm. In order to discriminate better between the different UV-absorbing zones in the isotachopherogram, ampholytes were used (p*I* 3–10; LKB, Bromma, Sweden). An aliquot of 1 μl of a 250-fold diluted stock solution of ampholytes was analysed together with 1–2 μl of sample.

The samples and the ampholytes were injected separately with different Type 701 10-μl microsyringes (Hamilton, Bonaduz, Switzerland) in order to avoid cross-contamination. Care should be taken with respect to the amount of ampholyte and sample; too much ampholyte will overload the system and the amount of sample should not increase the total length of ampholytes to an unacceptable extent. The ratio of the two depends on the sample and probably also on the inner diameter of the separation compartment.

In this study, we studied commercially available allergenic extracts in use for diagnosis and therapy of allergy, obtained from the Haarlems Allergenen Laboratorium (HAL), Haarlem, The Netherlands. In this particular application of isotachophoresis, it proved necessary to carry out a one-step sample pre-treatment. The allergens studied were dissolved in a buffer for several reasons. For injection purposes, the solutions should be isotonic and, in addition, the buffer is necessary for stabilization and maintenance of sterility. The resulting ionic strength limits the amount of sample that can be injected. Moreover, the phenol in the buffer solution disturbs the protein profiles. For these reasons, the samples were dialysed against water prior to analysis. Sample amounts of 100–200 μl could conveniently be dialysed in a dialysing tube of 5 mm I.D. bent into a U-turn, and fixed by a clamp. In a 4-l vessel with a magnetic stirring device, ten samples were dialysed simultaneously. The time of dialysis required was at least 3 h.

RESULTS AND DISCUSSION

Prior to analysis, the quality of the operational system was evaluated by means of a blank run. Both the length of the carbonate zone, migrating close to the leading electrolyte, and UV-absorbing impurities migrating between carbonate and the terminator are important in this respect. Only the carbonate originating from the terminating electrolyte will interfere, as it will dilute the steady-state zones migrating in between. The length of the carbonate zone (Fig. 1a) was *ca.* 20 sec at 15 μA and the day-to-day reproducibility was *ca.* 10%, provided the precautions mentioned above were taken.

In this pH 8 operational system, it is virtually inevitable that small amounts of UV-absorbing impurities will migrate between leading and terminating zones (Fig. 1a). Fortunately, however, this peak consists of a large number of constituents at low concentration. When analysing ampholytes only, the UV background is therefore relatively low. The actual profiles of the samples are superimposed on this background (Fig. 1b). The continuous mobility gradient is visible in the conductivity detector signal. This trace should not be mistaken for a low-resolution thermal detector signal. No information, other than the length of the carbonate zone, is obtained from this signal.

The day-to-day reproducibility of the protein profiles was evaluated by analysing a sample of house-dust mite extract, a solution of which was stored at -20°C in portions of 200 μl (this was a solution in water, not buffer). The resulting profiles, shown in Fig. 2, indicate satisfactory reproducibility when interpreted in a semi-quantitative manner. Exact calculation of peak areas would be premature, considering the limited linear dynamic range to be expected. The peak-height reproducibility was *ca.* 10%.

Allergenic extracts from different origins clearly show characteristic profiles (Fig. 3). Isotachophoretic profiling thus permits unequivocal characterization of samples. Extracts of different origin or manufacturer can consequently be compared.

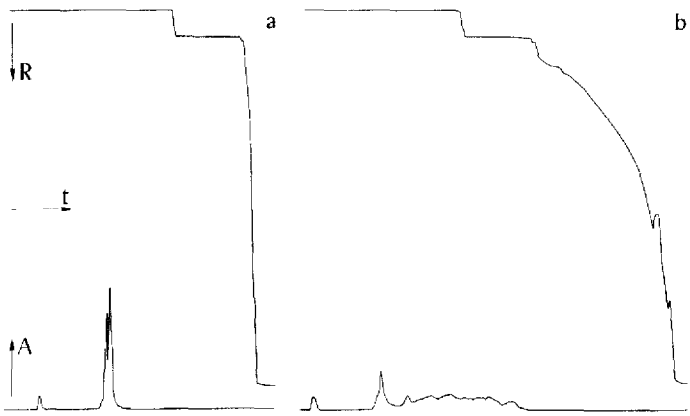


Fig. 1. Blank run of leading/terminator with conductivity (*R*) and UV detection (*A*) at 280 nm (a). When analysing ampholytes only, the UV-absorbing impurities form a background signal on which the sample profiles are superimposed. The continuous mobility gradient is visible in the conductivity detector signal (b).

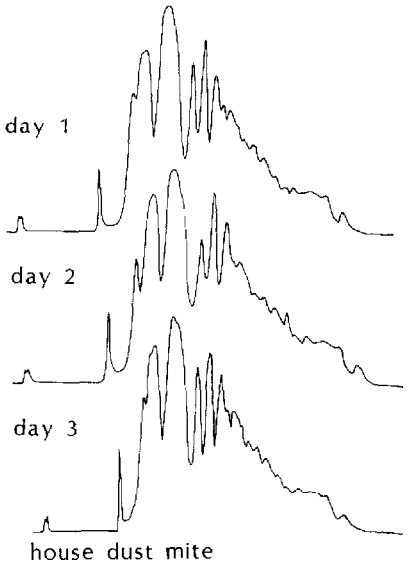


Fig. 2. Day-to-day reproducibility of the isotachophoretic analysis of a sample of house dust mite. The peak-height variation did not exceed 10%.

In contrast to zone length measurements in isotachophoresis, the signal amplitude obtained in the experiments described is a measure of amount. The method is therefore suitable for the determination of the concentration of certain, albeit unidentified, constituents.

As a patient is treated with increasing amounts of an allergenic extract during a prolonged period of time, storage in a refrigerator at 4°C is usual. In practice, the extracts will be removed from the refrigerator each time the patient receives an injection. In order to investigate the effect of the degradation to be expected as a result of such a procedure, the following experiment was designed: fresh extracts were analysed and kept at 4°C for 4 weeks and at 20°C for 4 h on each working day during these 4 weeks. After this period, the extracts were kept continuously at 20°C for a

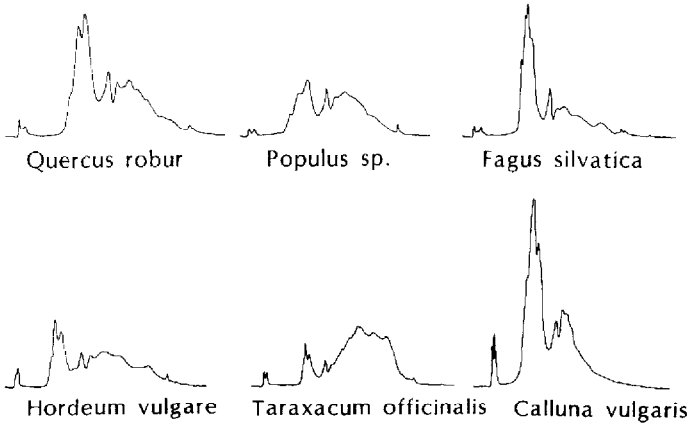
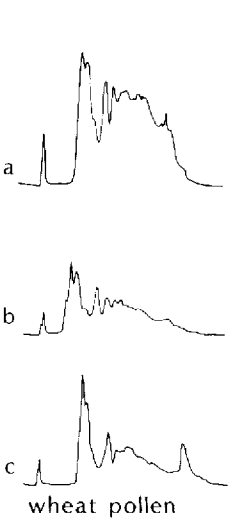


Fig. 3. Characteristic protein profiles of pollen extracts of different origin.



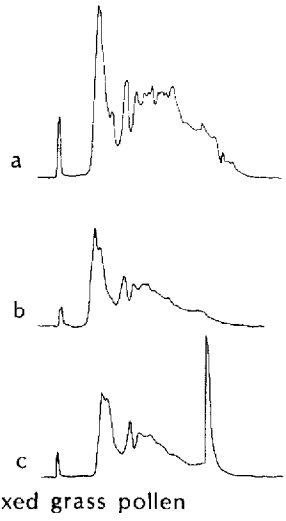


Fig. 4. Isotachophoretic profile of a wheat pollen extract. Fresh extract (a), after 4 weeks at 4°C and 4 h at 20°C each working day (b) and after an additional 1 week at 20°C (c).

Fig. 5. Isotachophoretic profile of a mixed grass pollen extract. Conditions as in Fig. 4.

further 1 week. The corresponding protein profiles are shown in Fig. 4 for wheat pollen and Fig. 5 for grass pollen. Both profiles show marked changes after 4 weeks of storage under "doctor' office" conditions. After a further 1 week at room temperature, which can be considered as forced degradation, the protein profiles show further changes, particularly the appearance of a UV peak of relatively low effective mobility. This peak, possibly a degradation product, was observed in a number of extracts of different origin.

CONCLUSIONS

Isotachophoresis is a practicable and reproducible inexpensive microtechnique for the characterization of allergenic extracts. In comparison with other techniques, *e.g.*, isoelectric focusing, the analysis time is short and the analytical data for a specific sample are directly available. The results suggest that isotachophoresis is convenient for the routine profiling of allergenic extracts, for both quality control and standardization.

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