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ISOLATION OF INFLUENZA VIRAL PROTEINS BY SIZE-EXCLUSION AND ION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: THE INFLUENCE OF CONDITIONS ON SEPARATION

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SUMMARY

Constituent proteins of influenza virus and of vaccines containing whole virus or viral antigens (surface antigen vaccines) have been separated by size-exclusion high-performance liquid chromatography. Preparations of whole virus have also been examined by high-performance anion-exchange chromatography. The separations are influenced by the conditions employed and, in particular, by the nature of detergent used to disrupt the virus and that incorporated in the eluting solvent. Individual proteins are recovered with retention of immunological activity. The method is applicable to small portions of a single human dose of vaccine.

INTRODUCTION

Influenza is unusual among viruses in that changes take place in the viral antigens in the strains circulating in the population. These changes may be relatively minor or major —when they may be associated with pandemics— and they have been identified at the structural level in both the neuraminidase[1] and haemagglutinin antigens[2]. Following disruption of virus with detergent and/or enzymes, various methods, some of which employ chromatography[3], have been used to isolate the constituent proteins. Recently, attempts have been made at separation by high-performance liquid chromatography (HPLC). Detergent-disrupted influenza virus has been examined by reversed-phase HPLC[4] but the recoveries of protein were low and only the matrix protein, which is known to be very stable, was obtained in an immunologically active form. Welling and colleagues have examined Sendai virus proteins by reversed-phase[5] and by anion-exchange[6] HPLC, the former leading to good recovery of one protein with lower recovery of others in purified form, and the latter giving good separations in the presence of detergent in the buffer, although some components were not retained on the column.

Because of the ease of recovery of proteins and glycoproteins in an immunologically active form after size-exclusion HPLC[7], we have used this technique to examine the effects of changes in conditions of preparation and chromatography on the separation of influenza viral proteins. In this paper we report some of our observations, including the recovery of several proteins with retention of activity in

single-radial immunodiffusion assays, and the examination of vaccines for human use. Less successful attempts have also been made to achieve useful separations by anion-exchange HPLC.

EXPERIMENTAL

Materials

A suspension of X-49 influenza virus was obtained as described[8], standardised to a content of 10 mg/ml, and provided by Dr. Oxford. Trivalent vaccines contained material from the strains A/Brazil/11/78, B/Singapore/22/79 and either A/Bangkok/1/79 (whole virus) or A/Philippines/2/82 (surface antigen vaccine) having approximately 10 μg haemagglutinin (HA) per strain (15 μg for the B strain in the whole virus vaccine) per 0.5 ml dose.

Buffer salts were of analytical grade, solvents of HPLC grade, remaining chemicals and detergents were from BDH (Poole, U.K.).

Apparatus

Two chromatographic systems were employed, (a) for isocratic analyses: Altex 110A pump, Rheodyne 7125 injection valve, Cecil 212A variable-wavelength UV detector, Tekman TE200 recorder and a Gilson TDC 80 fraction collector; (b) for gradient analyses: Spectra Physics SP 8700 solvent delivery system with SP 8750 organizer module, Cecil 2112 variable-wavelength UV monitor, remainder as above.

Operating conditions

The columns employed for separations by size exclusion were: TSK G4000SW, TSK G3000SW and TSK G5000PW, all of dimensions 300 × 7.5 mm I.D., from Toyo Soda (Tokyo, Japan). The mobile phase was 0.1 *M* sodium phosphate buffer, pH 7.0, containing 0.1% of one of the following detergents: sodium dodecyl sulphate (SDS), Brij 35 (polyoxyethylene-lauryl ether) or Lutensol ON 70D (a mixture of polyethylated linear aliphatic alcohols). The columns were calibrated with protein standards (mol.wt. 12,500–450,000) for each mobile phase. The flow-rate was 0.5 ml/min and detection was by UV absorption at 210 nm. Fractions of 0.5 ml were collected. Anion-exchange separations were performed using a TSK IEX 645 DEAE column of dimensions 75 × 7.5 mm I.D. (Toyo Soda, Tokyo, Japan) with a guard column (15 × 7.5 mm I.D.), and an Aquapore AX 300 column of dimensions 250 × 4.0 mm I.D. (Brownlee Labs, Santa Barbara, CA, U.S.A.).

Electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described by Oxford *et al.*[8] and the bands were located by silver staining using the method of Porro *et al.*[9].

RESULTS AND DISCUSSION

Influenza virus carries two major antigenic glycoproteins, haemagglutinin (HA) and neuraminidase (NA), on its surface and other proteins, including the matrix protein (M) and nucleoprotein (NP), within the membrane. The proteins are released

by treatment with detergent, and the haemagglutinin may be reduced with mercaptoethanol to its sub-units HA_1 and HA_2. After treatment of X-49 virus particles in suspension with a solution of detergent in buffer, the clear solution obtained was analysed by size exclusion on TSK G5000 PW and TSK G4000 SW columns. The latter gave superior resolution. Fig. 1a shows the profile on the G4000 SW column obtained using sodium dodecyl sulphate (SDS) for disruption of the virus and in the running buffer. The peak composition was studied by fractionating 500 μg of disrupted virus in the system shown in Fig. 1a. Portions of the fractions were examined by SDS–PAGE, the samples being treated with 2-mercaptoethanol and heated before application to the gel. The same fractions were examined, without treatment, by single-radial immunodiffusion (J. Oxford, unpublished). The results from both techniques show that the peak at 8.8 min (totally excluded) probably contains aggregated HA and other aggregates, that at 15.2 min is HA itself, that at 17.4 min is the matrix protein, completely separated from HA, and that at 19.6 min contains both NP and ovalbumin, the latter from the eggs in which virus was grown. The peak at 23.2 min gave no bands on SDS–PAGE and may contain fragments of one or more proteins which are too small to be recognised by the immunodiffusion assay. The elution position of NA has not been confirmed. The relationship of known molecular weight of the individual proteins to their elution position is under investigation. It is clear, however, that NP is retarded on the column. Unlike the reversed-phase separation of influenza virus[4], therefore, immunologically active HA, NP and matrix protein can be recovered from the size-exclusion column. In addition, the recovery of protein

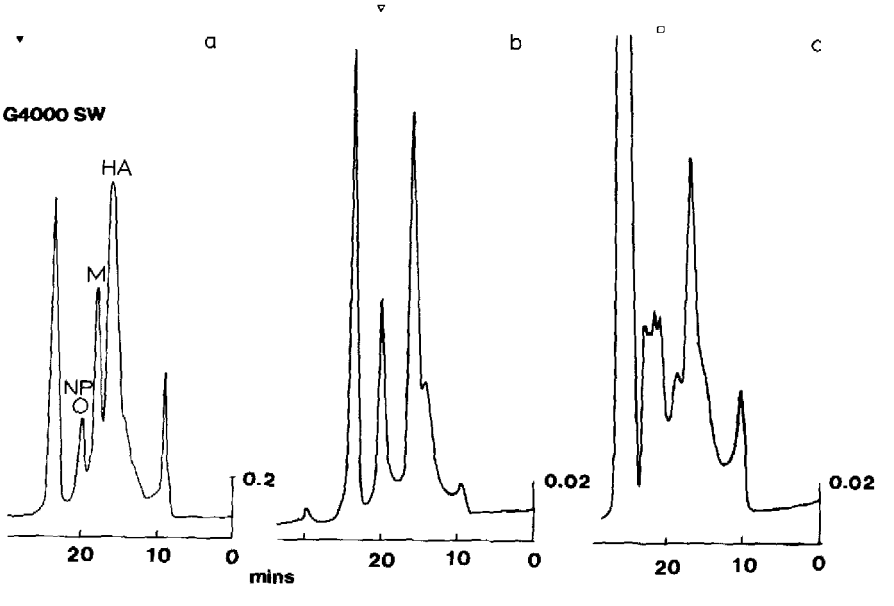

Fig. 1. Examination of influenza virus extracts by size-exclusion. Mobile phase: 0.1 *M* sodium phosphate, pH 7, containing 0.1% sodium dodecyl sulphate. Flow-rate 0.5 ml/min. UV detection at 210 nm. Column, TSK G4000 SW. (a) *Virus (100 μg) in 2% SDS.* (b) *Sample (10 μg) in 10%* Lutensol in buffer. (c) Sample 20 μl (*ca.* 10 μg), dissolved with Brij 35. HA = haemagglutinin; M = matrix protein; NP = nucleoprotein; O = ovalbumin.

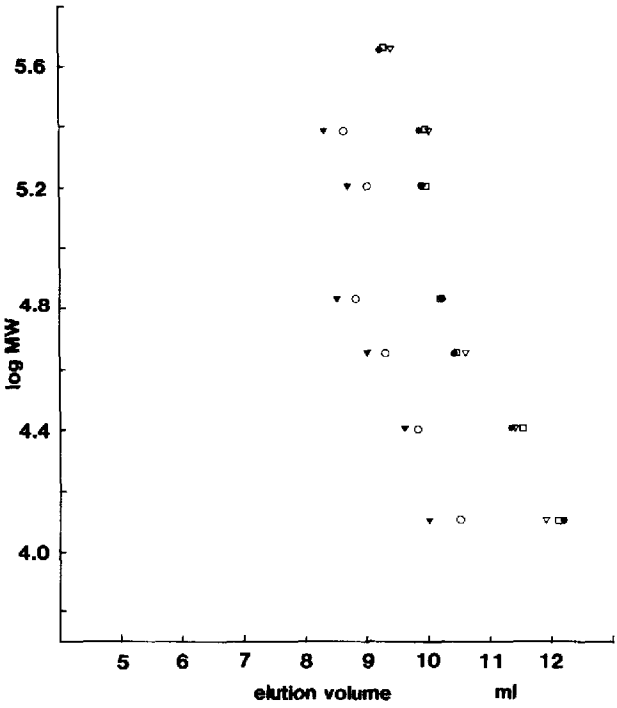

Fig. 2. Calibration of TSK G4000 SW column with standard proteins. Mobile phase 0.1 *M* sodium phosphate containing 0.1% detergent. Detergents: (▼) SDS, (▽) Lutensol, (□) Brij, (●) no detergent. Samples dissolved in same detergent as for buffer except: (○), sample in Lutensol, buffer detergent SDS. Other conditions as for Fig. 1. The proteins examined were cytochome *c* (MW 12,500), chymotrypsinogen A (24,000), ovalbumin (45,000), bovine serum albumin (68,000), aldolase (158,000), catalase (240,000) and ferritin (450,000).

is greater than 90%. If the virus is disrupted with a non-ionic detergent, such as Lutensol (Fig. 1b) or Brij (Fig. 1c), the elution profiles in the SDS–buffer system differ significantly from that in Fig. 1a and from each other. These differences in the profiles may reflect variations in the abilities of the individual detergents to disrupt the virus itself and the oligomeric structures of its constituent proteins (for example, HA is a trimer and NA a tetramer).

To examine further the influence of detergent on the elution profile, the behaviour of the standard calibration proteins was tested (Fig. 2). The proteins were dissolved in a solution of detergent and buffer which was immediately injected into the G4000 SW column. The non-ionic detergents, Brij and Lutensol, had no effect on the elution positions of the proteins compared with those without detergent. The binding of detergent, therefore, does not appear to affect the size of the molecule in these instances. By contrast, SDS consistently causes a reduction in elution volume, indicating that the proteins adopt a more open molecular configuration. When samples were dissolved in Lutensol, but chromatographed in SDS, the elution positions shifted, but not as much as when SDS was also used for disruption. It appears that the presence of SDS in the buffer has an effect on the protein structure additional to that of Lutensol.

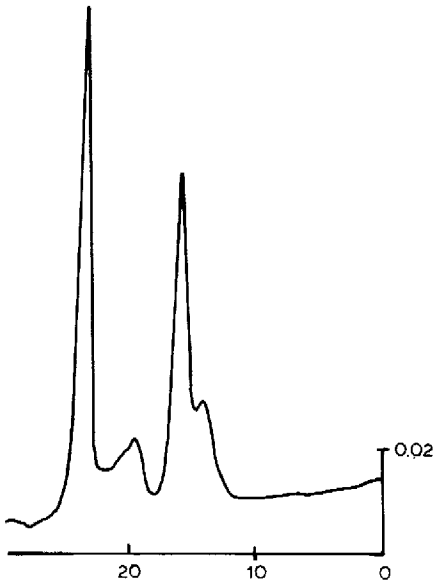

Fig. 3. Separation of influenza viral proteins, extracted and chromatographed in the presence of the non-ionic detergent Lutensol. Column: TSK G4000 SW. Mobile phase 0.1 *M* sodium phosphate, pH 7, containing 0.1% Lutensol. 10 μg virus applied in 10% Lutensol. Other conditions as Fig. 1.

A similar effect is observed with influenza virus. Virus disrupted with and chromatographed in Lutensol gives an elution profile (Fig. 3) which differs significantly from that in Fig. 1a, where the virus has been exposed to and chromatographed in SDS, but resembles that in Fig. 1b. However, because of the observations shown in Fig. 2 with the standard proteins, the profiles may not be directly comparable: the appearance of the more excluded peak in Fig. 1b at approximately 9.6 min may not indicate reaggregation of viral proteins but a further unfolding of one or more components by the action of SDS.

Fig. 4 shows the results obtained when three influenza vaccines, one of whole virus (Fig. 4a) and two of purified surface antigens (Fig. 4b and c), were examined by the same system as that for Fig. 1a. The vaccines contain a minimum 30 μg HA per 0.5 ml dose (whole virus 35 μg). The two purified antigen vaccines contain predominantly HA and NA but the amounts of other constituents differ. The vaccines were sampled directly from the final container and chromatographed without pretreatment. The amount applied to the G4000 SW column was equivalent to 4% of a single human dose in Fig. 4a and c and 8% in Fig. 4b. The elution profile in Fig. 4a is essentially similar to that in Fig. 1a, the broadened envelope between 10 and 20 min probably arising from the multivalent nature of the vaccine. The large peak at 30 min is given by the preservative (thiomersal). For Fig. 4a and b, the ratio of the HA peaks at 16 min reflects the difference in sample sizes. The chromatogram in Fig. 4b with a sharper profile between 12 and 18 min, shows a narrower molecular weight distribution of the major proteins in this vaccine than in the whole virus vaccine. However, the vaccine shown in Fig. 4c not only appears to contain relatively more HA than that in Fig. 4b but also a higher level of other constituents except those in

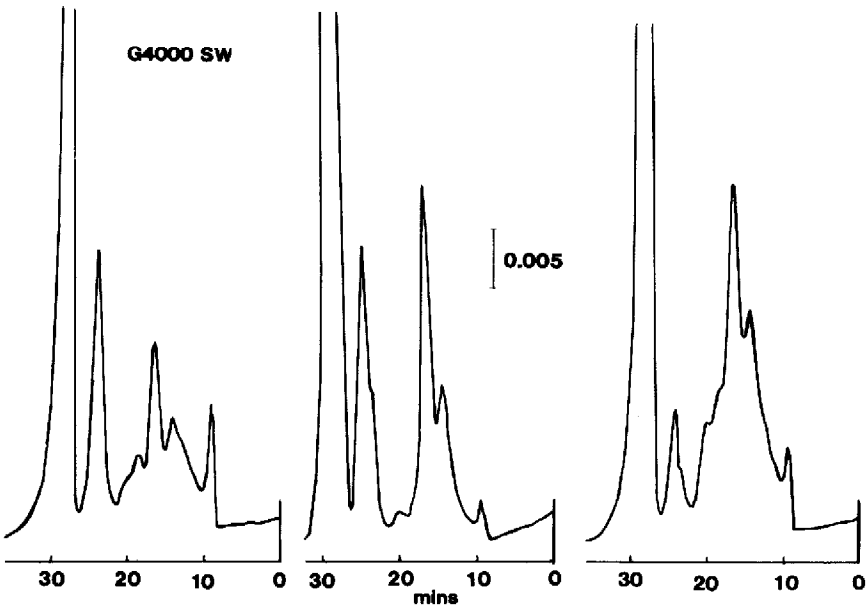

Fig. 4. Analysis of commercial vaccines (for composition see text). Column: TSK G4000 SW. Other conditions as Fig. 1. (a) Vaccine from whole virus (supplier 1); nominal HA present 1.4 μg. (b) 'Surface antigen' vaccine (HA and NA) (supplier 1); nominal HA 2.4 μg. (c) 'Surface antigen' vaccine (supplier 2); nominal HA 1.2 μg.

the peak at 23.6 min, and in this respect it more resembles the vaccine in Fig. 4a.

Following the successful isolation[6] of the fusion protein from Sendai virus by anion-exchange chromatography in the presence of the non-ionic detergent Triton X-100 (iso-octylphenoxypolyethoxyethanol), we attempted to separate some of the influenza virus proteins by a similar procedure. Disrupted whole virus and fractions from the size-exclusion separation were examined. Despite evaluation of numerous gradient systems and alterations in buffer composition, the results were disappointing. Fig. 5a and b show the profiles obtained with different anion-exchange columns under comparable conditions. The silica-based Aquapore column (Fig. 5a) gave sharp peaks but the recovery of material was poor. The IEX column having an organic support[10] and a different geometry retained the sample, and fewer components were resolved (Fig. 5b). The best separation on the latter column was achieved at higher pH (Fig. 5c) when a component eluted after 42 min which was not eluted at pH 5.5. In this case the virus was disrupted with SDS and was eluted from the column with a sodium chloride gradient. Methanol was present to prevent the aggregation of proteins. The extract was eluted as mainly a single peak with some smaller components. The position of the main peak was, however, hardly affected by the difference in conditions.

In conclusion, proteins from disrupted influenza virus have been separated by size-exclusion HPLC and recovered with activity in immunodiffusion assays. The results obtained suggest that this may be a valuable method for purification of viral components for the production of the specific antisera required to quantitate such assays for testing potency. The method can also be applied directly to amounts as

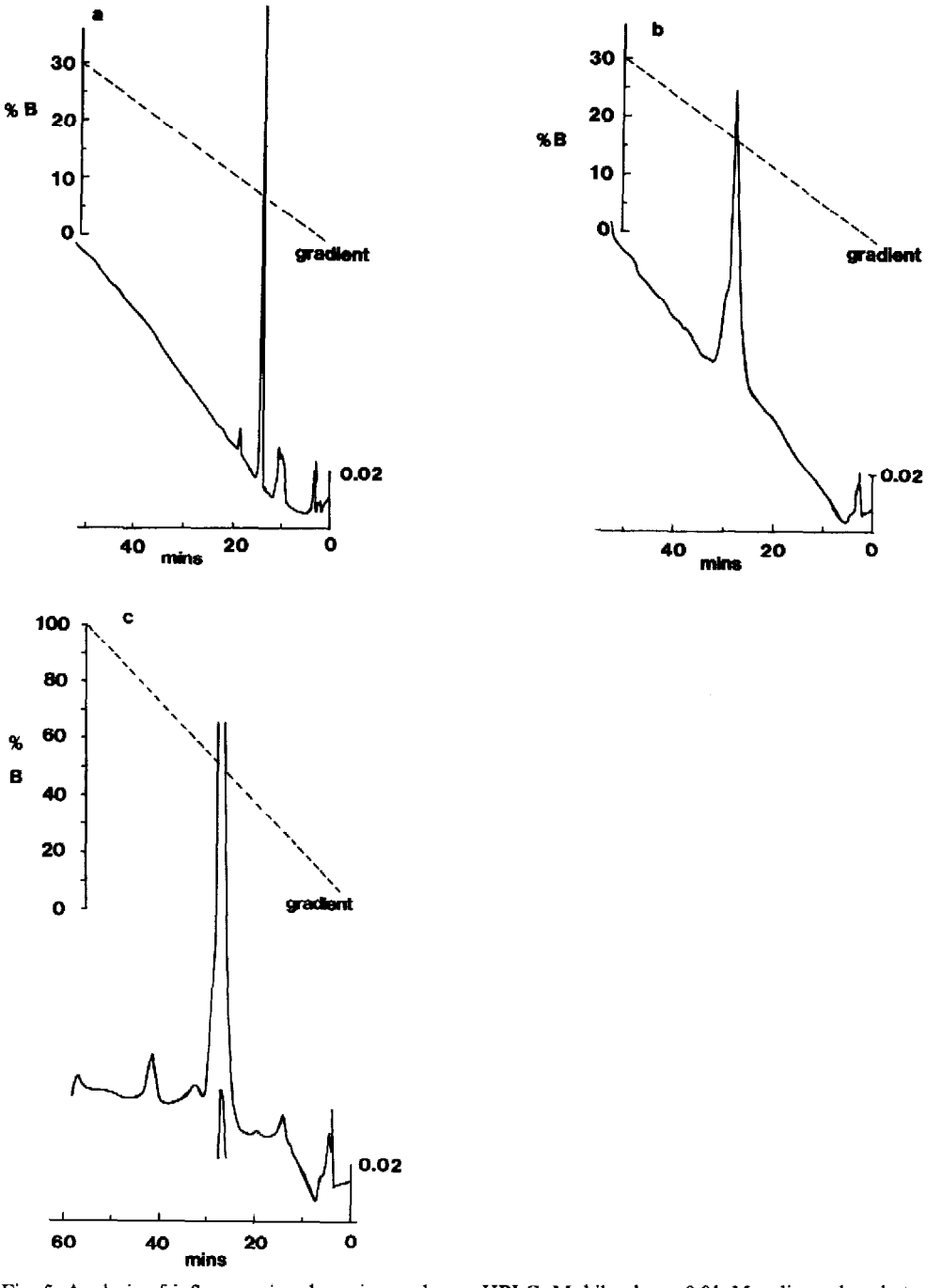

Fig. 5. Analysis of influenza virus by anion-exchange HPLC. Mobile phase: 0.01 *M* sodium phosphate pH 5.5. Gradient: 0–1 *M* NaCl over 50 min. Flow-rate: 1.0 ml/min. UV detection at 210 nm. (a) Column: Aquapore AX-300 (250 × 4 mm I.D.). Influenza virus (15 μg) in buffer containing 2% SDS. (b) Column: TSK IEX-645 DEAE (75 × 7.5 mm I.D.). Virus (20 μg) in buffer containing 2% SDS. (c) Column: TSK IEX-645 DEAE. Mobile phase: 0.01 *M* Tris–HCl (pH 8)–Methanol (60:40). Gradient: 0–0.3 *M* NaCl over 50 min. Influenza virus (70 μg) applied in mobile phase containing 2% SDS.

small as 4% of a single human dose of vaccine. Separations are influenced by the conditions under which the virus is disrupted and chromatographed, in particular by the detergent employed. Attempts to recover individual influenza viral proteins by anion-exchange chromatography have been less successful.

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