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High-performance liquid chromatographic approach to the separation of antiviral and immunostimulant fractions in Neuramide

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ABSTRACT

Neuramide, a tissue extract having antiviral action against influenza A virus and immunostimulant action, was analyzed by preparative size-exclusion high-performance liquid chromatography (HPLC) and a low-molecular-weight fraction responsible for the antiviral action was isolated after reversed-phase HPLC. Four fractions having immunostimulant activity were also isolated, as evidenced by their potentiating action in the human lymphocyte proliferation induced by phytohaemagglutinin.

INTRODUCTION

Neuramide (NMD) (Difa Cooper, Caronno Pertusella, Italy), a viral inhibitor that is present in crude preparations of tissue extracts which is active against herpes viruses, has been described previously[1,2]. It is also active against a wide range of viruses which are not herpes viruses, and one inhibitory activity of NMD act to block the adsorption of a variety of viruses[3,4]. NMD is also active against influenza A viruses *in vitro* via a block in the replication cycle after the virus has been adsorbed, and ultrafiltration experiments showed that the anti-influenza virus activity was concentrated in the material of molecular weight below 500 Da[5]. During these studies, an immunostimulatory activity was also shown[6,7].

Here we report the preparative high-performance liquid chromatographic (HPLC) analysis of the antiviral and the immunostimulant fraction in NMD.

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EXPERIMENTAL

Neuramide (NMD) preparations were obtained from Difa Cooper.

Ultrafiltration experiments

NMD was fractionated by molecular sieving through Amicon type UM, YM and PM membranes. Markers were actinomycin D (MW = 1225); eosin yellow (MW = 691) and bromphenol blue (MW = 173).

HPLC analyses

Preparative size-exclusion chromatography (SEC) was performed by dissolving the lyophile in distilled water to obtain a concentration of 70 mg/ml. Samples were injected through a 1.1-ml loop. The instrument was a Varian 5000 HPLC system (Varian, Palo Alto, CA, U.S.A.) equipped with a TSK G2000 SWG size-exclusion column (60 cm × 21.5 mm I.D.) (Toyo Soda, Tokyo, Japan) eluted with a two-step gradient (45 min distilled water and 45 min 0.1 *M* sodium chloride solution) at a flow-rate of 6 ml/min. The detector was a Model 1040 diode-array detector (Hewlett Packard, Palo Alto, CA, U.S.A.). Fractions were tested for antiviral activity against the influenza virus and for immunostimulant activity by observing the enhancement of the human lymphocyte proliferation induced by phytohaemagglutinin.

Analytical reversed-phase HPLC of fractions from preparative runs was performed by dissolving the lyophilized material in 0.05 *M* ammonium acetate buffer (pH 6.5) and injecting through a 10-μl loop (30 μg per injection). The instrument was a Model SP8800 system (Spectra-Physics, San Jose, CA, U.S.A.), equipped with a Supelcosil PLC-18 reversed-phase column (25 cm × 4.6 mm I.D.) (Supelco, Bellefonte, PA, U.S.A.), eluted with 0.05 *M* ammonium acetate buffer (pH 6.5) at a flow-rate of 1 ml/min. The detector was a Model 484 tunable absorbance detector (Waters Assoc., Milford, MA, U.S.A.).

Isocratic reversed-phase HPLC purification of fractions from size-exclusion HPLC preparative runs was performed by dissolving the lyophilized material in water–acetonitrile (1:9) and injecting the solution through a 10-μl loop. The instrument was a Spectra-Physics SP8800 system equipped with a Waters Assoc. μBondapak-NH_2 column (30 cm × 3.9 mm I.D.), eluted with water–acetonitrile (1:9) at a flow-rate of 0.8 ml/min. The detector was a Waters Assoc. Model 484 tunable absorbance detector.

Extraction of antiviral components with methylene chloride

An aliquot of 252 mg of the mixture A + B + sodium chloride obtained by ultrafiltration–size exclusion HPLC (Fig. 1) was dissolved in 50 ml of water and extracted three times with 50-ml portions of methylene chloride. The organic extracts were collected, dried over sodium sulphate and the solvent was evaporated under reduced pressure to give 9 mg of the mixture A + B.

Elemental analysis

Carbon and nitrogen contents were measured using a Perkin-Elmer 240 elemental analyser.

Antiviral assay

Influenza A virus strains H_0N_1 and H_2N_2 were grown in chicken embryos and maintained at −70°C until they were used. Titration of the anti-influenza virus effects of NMD or chromatographic fractions was carried as follows. Serial dilutions of the samples in 0.1-ml volumes were added in triplicate to 96-well microtitre cultures of canine kidney cells (MDCK cells), then 0.025 ml of influenza A virus was added to give an input multiplicity of infection of 10 PFU per cell. Supernatants were collected and virus yields were determined after 48 h by a haemagglutination assay, which was performed in triplicate[8]. The end-point activity was defined as the last dilution that was capable of inhibiting the haemagglutination yield four-fold, compared with the virus control yield.

The standard assay was adapted to study the effect of the chromatographic fractions on influenza A virus replication by addition of the inhibitor to the MDCK cell monolayers after the virus challenge. At the high multiplicity of infection which we used, all the cells were infected after 1 h of incubation at 37°C.

Fractions diluted 1:32 and 1:64 in Eagle minimum essential medium, containing 2% foetal calf serum, which were well within the active range of the material, were tested to evaluate the possible toxic effects of NMD or the fractions. The viability of the monolayers after incubation for 48 h in the presence of NMD or the fractions was evaluated by Blue Dye exclusion and by current criteria.

Proliferation assay

Lymphocytes were isolated from heparinized peripheral blood of healthy donors, as described previously[9].

Mononuclear cells were resuspended at a final concentration of $1 \cdot 10^6$ cells/ml in RPMI 1640 medium, supplemented with 10% foetal calf serum, glutamine (2 m*M*) and gentamicin (50 μg/ml). Each sample was stimulated in triplicate microwell cultures in the presence of phytohaemagglutinin (PHA-M, Gibco) at a concentration of 12.5 μg/ml and at three different dilutions (1:100, 1:500 and 1:10 000) of NMD fractions. After incubation for 48 h at 37°C in a humidified atmosphere of 5% carbon dioxide in air, the cultures were labelled for 21 h with 0.5 μCi/well of tritiated thymidine (2 Ci/mmol, Amersham) and were then harvested onto glass-fibre strips with a multiple automated harvester (Skatron), included in scintillation fluid and counted.

RESULTS AND DISCUSSION

The first chromatographic enrichment procedure was performed with material having both antiviral and immunostimulating activity. The antiviral activity had been found in the ultrafiltered fraction containing components of MW less than 500 Da[5] and the immunostimulant activity in the ultrafiltered fraction below 5000 Da[6,7]. Hence, ultrafiltered material with a 5000-Da cut-off was used. In the SEC–HPLC procedure, the components were first eluted with water, then with 0.1 *M* sodium chloride solution and the fractions were tested for biological activity. The immunostimulating activity was tested by monitoring the potentiating effect of the fractions in the human lymphocyte proliferation, induced by phytohaemagglutinin. Several controls were used in this test, owing to the possibility of differences in the

responsiveness of individuals. For the fraction eluted with saline, this was dialysed and lyophilized before the biological test. Fig. 1 shows the chromatographic profile and the histogram of the immunostimulating activity for three dilutions of the chromatographic fractions. Four chromatographic regions of biological activity appear in most controls. The most prominent region (23% of the original amount of material) is the first to be eluted, and is composed of material at MW $\approx$ 5000 Da. A second, less well defined region (13% recovery) appears in the bulk of the chromatographic peak, and the third region occurs in the tail of the peak (6% recovery) and probably contains a small amount of active low-molecular-weight material. Some active material is retained by the column and is eluted with 0.1 *M* sodium chloride solution. These results suggest that several families of components in the extract have immunostimulating activity.

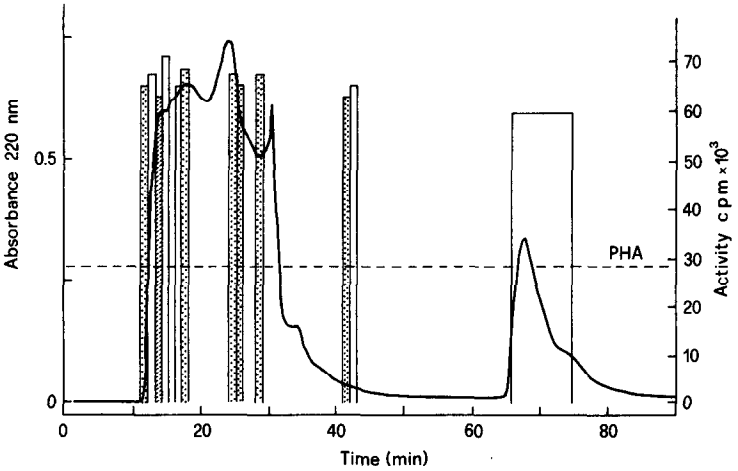

Fig. 1. SEC–HPLC analysis of NMD ultrafiltered with a 5000-Da cut-off membrane and histogram of the enhancement of the human lymphocyte proliferation induced by phytohaemagglutinin at three dilutions (white, 1:100; dotted, 1:500; dashed, 1:10 000).

All fractions were also tested against influenza virus in order to evaluate the presence of antiviral components. It could be shown that these were concentrated in the material eluted by 0.1 *M* sodium chloride.

The fact that the antiviral components were eluted after the V_t value suggested that some effect other that size exclusion was occurring, *i.e.*, some interaction of these components with silica. This observation could be used to improve the purification procedure by elution of non-antiviral components with water before the elution of the antiviral fraction with 0.1 *M* sodium chloride solution.

As antiviral components had MW below 500 Da, the ultrafiltrate below 500 Da having a specific activity of 2.28–4.57 activity units$^{5.8}$/mg was injected into a preparative SEC–HPLC column; the result is shown in Fig. 2. All chromatographic fractions were lyophilized, dissolved in methanol and filtered and the methanolic extract was evaporated to dryness, weighed and submitted to evaluation of the antiviral activity. This was concentrated in the fraction eluted with 0.1 *M* sodium chloride. The specific

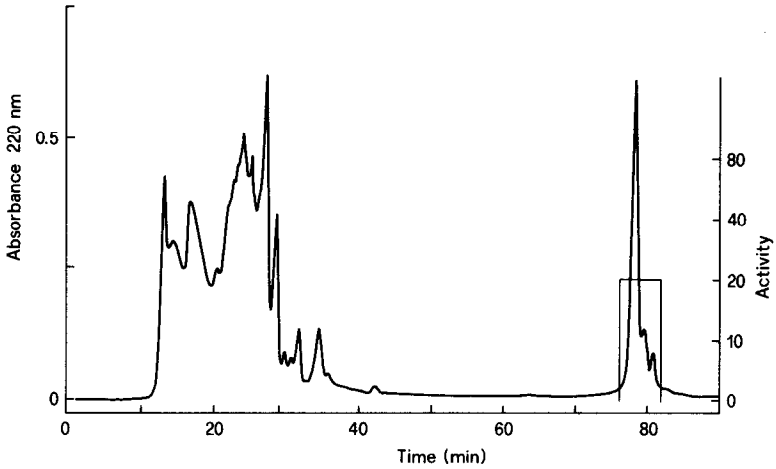

Fig. 2. SEC–HPLC analysis of NMD ultrafiltered with a 500-Da cut-off membrane.

activity of this material was 6.25–12.5 activity units[5,8]/mg with a six-fold increase in comparison with the original ultrafiltrate. Much sodium chloride was present in mixture with the organic material.

The biologically active fraction consisted of two peaks. The diode-array spectrophotometric analysis of these showed a strong absorption at 220 nm and had a very low absorption at 254 nm. Thus, the active fraction seemed not to contain nucleic bases or aromatic amino acid-containing peptides but to be constituted of sugars, non-aromatic amino acid-containing peptides or glycopeptides.

The material containing the two peaks in SEC–HPLC was then analysed with a different chromatographic procedure. Reversed-phase HPLC on a C_{18} column (RP-HPLC-C_{18}) with elution by 0.05 *M* ammonium acetate buffer (pH 6.5) and monitoring at 220 nm gave the result shown in Fig. 3. Again, two components were

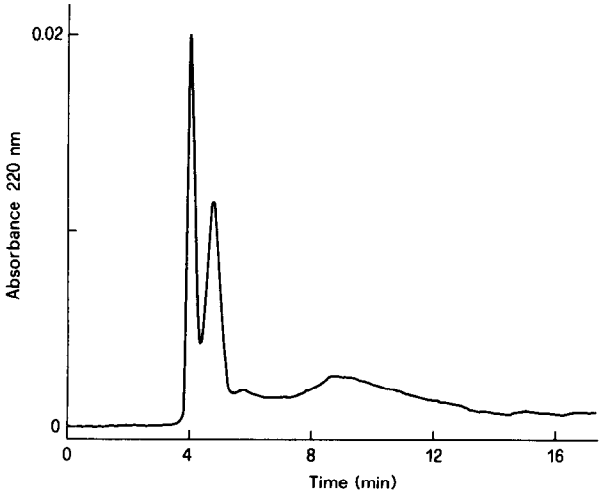

Fig. 3. RP-HPLC-C_{18} analysis of the antiviral fraction from the SEC–HPLC separation.

eluted after a short retention volume. This indicated that they were very polar products.

Reversed-phase HPLC was a good separation procedure for these two components. A better resolution was obtained using an amino-bonded RP-HPLC column (RP-HPLC-NH_2) eluted with water–acetonitrile (1:9). The elution profile typical of very polar material obtained by monitoring at 220 nm is shown in Fig. 4.

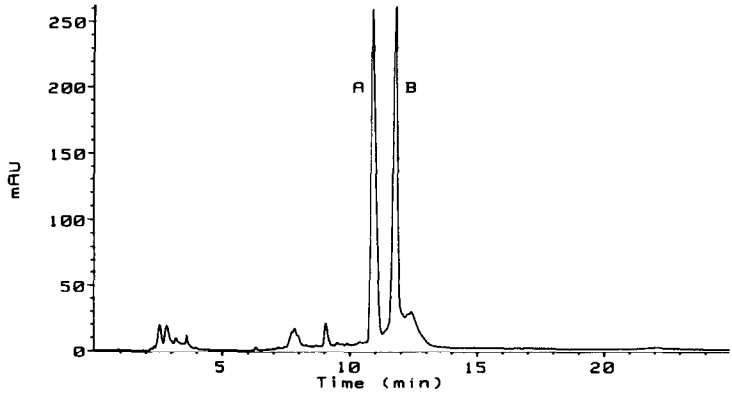

Fig. 4. RP-HPLC-NH_2 analysis of the antiviral fraction from the SEC–HPLC separation.

SEC was repeated fourteen times and the antiviral fraction was collected; 639 mg of material were obtained from 980 mg of NMD ultrafiltered below 500 Da. As this material was shown by the elemental analysis to contain only 4% of organic matter and much sodium chloride, an aliquot of this was used for the solvent extraction procedure intended to eliminate the salt and calculate the enrichment obtained.

Further 3-mg aliquots were injected into the RP-HPLC-NH_2 column and this procedure was repeated twenty times, which made it possible to obtain enough material for the characterization of the individual antiviral components. The lyophiles obtained from both peaks (compounds A and B) were submitted to elemental analysis. The high content of sodium chloride allowed only the carbon to nitrogen ratio in these samples to be obtained; this was 3:2 for compound A and 5:2 for compound B. These data and the UV absorption spectra suggested a polar aliphatic peptidic nature for these compounds.

Compounds A and B could be obtained from their mixtures with sodium chloride by extraction with methylene chloride. This allowed it to be calculated that a nearly equimolecular mixture of compounds A and B was present in a 2% amount in the original NMD ultrafiltrate below 500 Da.

In conclusion, enrichment of the antiviral activity of NMD against the influenza A virus may be obtained by an ultrafiltration and preparative SEC–HPLC sequence. This allows the isolation of two very polar components which may be further purified by RP-HPLC and solvent extraction for future structure determination studies. Four chromatographic fractions having immunostimulant activity have been also obtained by preparative SEC–HPLC.

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