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High-performance liquid chromatographic purification of antiviral components in Neuramide

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ABSTRACT

Neuramide (NMD), a tissue extract having antiviral action against influenza A virus, was analysed by preparative size-exclusion high-performance liquid chromatography followed by solvent extraction and reversed-phase high-performance liquid chromatography. Some small peptides responsible for the antiviral action were isolated and their amino-acid content was determined.

INTRODUCTION

Neuramide (NMD) is a viral inhibitor that is present in crude preparations of tissue extracts. It is active against herpes viruses [1]. Ultrafiltration experiments have shown that the anti-influenza virus activity is concentrated in the material of molecular weight below 500 dalton [2].

In a previous paper [3] we reported the use of a sequence of chromatographic steps for the enrichment of antiviral and immunostimulant components in NMD. Here we report the preparative high-performance liquid chromatographic (HPLC) multi-step separation of the antiviral fraction in NMD.

EXPERIMENTAL

NMD preparations were obtained from Difa Cooper (Caronno Pertusella, Italy).

HPLC analyses

Preparative size-exclusion HPLC (HPSEC) and tests of antiviral activity were performed as reported previously [3]. Analytical reversed-phase HPLC (RP-HPLC) of the antiviral fraction from HPSEC was performed after methylene chloride extrac-

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tion. Methanol solutions were injected through a 20-μl loop (50 μg per injection). The instrument was a Varian (Palo Alto, CA, USA) Model 5000 system equipped with a LichroCART 100 RP-18 (5 μm) RP column (25 cm × 4.6 mm I.D.) (Merck, Darmstadt, Germany) eluted with methanol at a flow-rate of 1 ml/min. The detector was a Hewlett-Packard (Palo Alto, CA, USA) Model 1040 diode array.

Preparative RP-HPLC purification of the antiviral fraction from HPSEC was performed after methylene chloride extraction. Solutions in water–acetonitrile (2:8) were injected through a 100-μl loop. The Varian 5000 system was used, equipped with a Waters Assoc. (Milford, MA, USA) μBondapak-NH_2 column (30 cm × 3.9 mm I.D.) eluted with water–acetonitrile (2:8) at a flow-rate of 4 ml/min. The Hewlett-Packard Model 1040 diode-array detector was used.

Extraction of antiviral components with methylene chloride

An aliquot of 1.9 g of the mixture obtained by elution with 0.1 *M* sodium chloride in eight runs of the sequence ultrafiltration–HPSEC and shown to have antiviral activity 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 7.9 mg of mixture. Antiviral activity was present in both the methylene chloride extract and the aqueous phase.

RESULTS AND DISCUSSION

The HPSEC procedure reported in previously [3] was performed sixteen times, in order to obtain sufficient material for further purification procedures. The antiviral material was eluted with sodium chloride solution and further purification was required to separate the organic material from sodium chloride. This was performed by methylene chloride extraction. Part of the antiviral activity was thus transferred into the organic phase, and was freed from sodium chloride.

The organic extract was then analysed by C_{18} RP-HPLC (RP-HPLC-C_{18}), eluting with methanol and monitoring at 220 nm (Fig. 1). Two peaks were obtained.

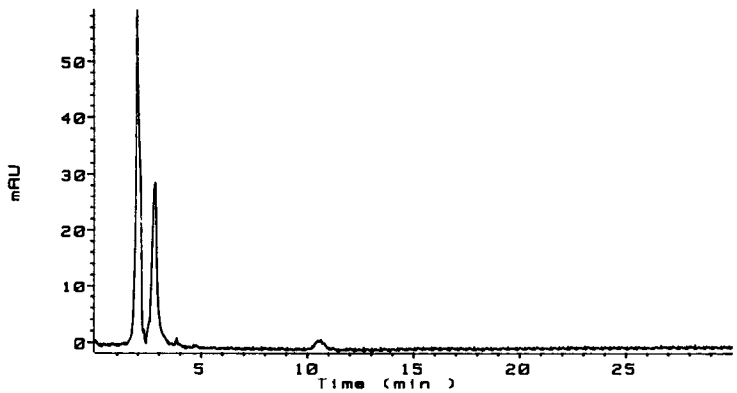

Fig. 1. RP-HPLC-C_{18} analysis of NMD after HPSEC and solvent extraction.

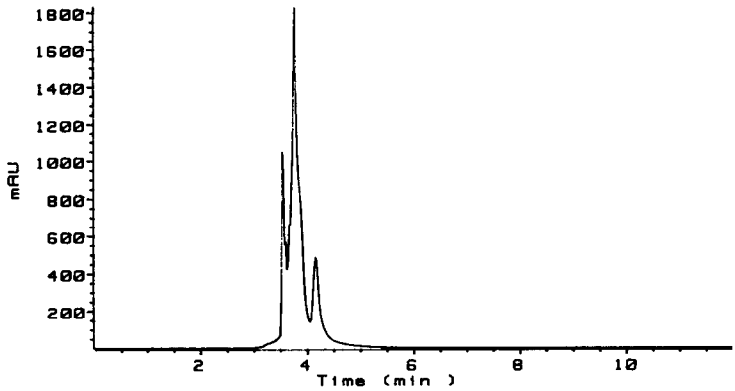

Fig. 2. RP-HPLC-NH_2 analysis of NMD after HPSEC and solvent extraction.

This procedure is unsuitable for scaling-up of the purification as injection of a large amount of material resulted in loss of resolution.

A second RP-HPLC procedure was then performed using an amino RP-HPLC column (RP-HPLC-NH_2) eluted with water–acetonitrile (2:8). An elution profile typical of very polar material was again obtained by monitoring at 220 nm. The result is shown in Fig. 2. At least three components were present, and the separation efficiency allowed the chromatography to be scaled up.

Four runs allowed sufficient material to be collected to perform the biological test which confirmed the antiviral activity of this mixture of components.

The amino acidic nature of the antiviral mixture was demonstrated by the analysis of its amino acid content. This analysis showed the presence of glycine, serine, threonine, glutamic acid and aspartic acid.

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