Isolation and Fast Purification of Neocarzinostatin by FPLC-Ion Exchange Chromatography

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Z. Naturforsch. **38 c**, 939–942 (1983); received July 1/August 31, 1983

Zinostatin, Antitumor Antibiotic, Fast Purification, Biological Activity, Protein-HPLC

Neocarzinostatin, a highly toxic antitumor protein containing an essential nonprotein chromophore, can be isolated and purified from culture filtrates of *Streptomyces carzinostaticus*. Usually a lengthy procedure of up to 60 h is necessary for the isolation, including several chromatographic steps partly under conditions which favour inactivation of the drug by release of chromophore. We describe a new method yielding practically clinical grade Neocarzinostatin from crude extracts in 20 min. This very fast and reproducible method was made possible by using a Mono Q anion exchange column filled with monodisperse gel material which has been recently developed.

Introduction

Neocarzinostatin (NCS) is an antitumor protein antibiotic. For clinical grade material we use in this communication the international nonproprietary name (INN) of the drug, zinostatin. It was first isolated from culture filtrates of *Streptomyces carzinostaticus* by Ishida [1]. The protein has a molecular weight of 10700 and consists of a single chain of 109 amino acids of known sequence [2]. It also contains an essential nonprotein chromophore [3, 4].

Work from several laboratories indicates that NCS causes DNA strand breaks by degrading the backbone sugars [5–7] via reactions of the chromophore [8–11].

Several independent investigations have shown that dissociation of the chromophore from protein and interaction with DNA are the first steps in NCS action [12, 13]. This dissociation process opens a new possibility for applications of NCS. The drug could be linked to monoclonal antibodies against surface antigens of tumor cells via bifunctional agents, to yield selective drug molecules [14, 15]. To accomplish such work quantities of almost a gram of biologically active NCS are necessary. Since stocks of clinical NCS appear to be exhausted several laboratories have turned to producing their own material [16].

However, most purification methods for NCS described in the literature employing liquid chromatography are rather tedious and involve many steps partly under conditions, which favour the transition of NCS to biologically inactive apo-NCS by release of chromophore [17–19]. The separation of active NCS from apo-NCS proved to be especially difficult and lengthy in our hands.

For these reasons we have adapted HPLC-techniques to purify NCS with newly developed column material which is commercially available.

In this communication we report a fast purification method for NCS from crude extracts. The activity of the drug isolated by fast protein liquid chromatography (FPLC) was measured by biological and physicochemical methods.

Materials and Methods

Chromatography

A high performance liquid chromatography system (Pharmacia FPLC) was employed for all the studies reported here. The system consists of two P-500 pumps, the gradient programmer GP-250, a fixed wavelength detector UV-1 (280 nm), and a two channel recorder REC-482 for the simultaneous recording of salt gradient and optical density. Fractions were collected by FRAC-100. A Mono Q HR 5/5 anion exchange column (Pharmacia) was used. The charged group on the monodisperse gel particles is $-CH_2-N^+-(CH_3)_3$. The column

Abbreviations: Apo-NCS, Neocarzinostatin free of chromophore and biologically inactive; FPLC, fast protein liquid chromatography; HPLC, high pressure liquid chromotography or how proteins like to be chromotographed; NCS, Neocarzinostatin.

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0341-0382/83/1100-0939 $ 01.30/0

capacity is about 25 mg protein. Samples were eluted from the column at a flow rate of 2 ml/min maintaining a pressure of 20 bar (2 MPa). The separation time was 16 min. The solvent A (start buffer) was 20 mM ammonium acetate, pH 5. Solvent B (limit buffer) was start buffer containing 1 M NaCl.

The gradient programmer was set as follows: a linear gradient of 0–3% B in 2.5 min, followed by 3–25% B in 11 min, and finally by 25–100% B in 2 min.

For gel filtration a Sephadex G-50 fine column (2.6 × 86 cm) equilibrated with 20 mM ammonium acetate, pH 5, was used. Material was eluted from that column with a flow rate of 26 ml/h.

Neocarzinostatin

Crude extracts of NCS were obtained from culture filtrates of *Streptomyces carzinostaticus* by using the methods described in the literature [21, 23]. Enriched crude extract was prepared by pooling biologically active fractions after gel filtration of crude extracts on Sephadex G 50. Clinical zinostatin which originated from Kayaku Antibiotics Inc. was a gift of the Investigational Drug Branch of the National Cancer Institute Bethesda, Md, USA. It was stored at 4 °C in 15 mM sodium acetate buffer, pH 5 at a concentration of 660 µg/ml.

All preparative steps with crude extract or zinostatin were done under conditions which minimize the transition from NCS to apo-NCS (pH 5, temperature at 4 °C, protected from daylight). For some experiments NCS was converted into apo-NCS by UV irradiation (335 nm).

NCS concentrations were determined by fluorescence spectroscopy [8] and biological assay.

Quantitative NCS determination by fluorescence spectroscopy

Fluorescence spectroscopy was done with a Perkin-Elmer 650-10S spectrometer. To NCS-solutions in phosphate buffer 20 mM, pH 7.5, isopropanol was added (final concentration about 4 M) to accelerate the dissociation of chromophore from NCS [8]. The released chromophore "decays" in aqueous solutions giving an inactive product with a distinct fluorescence at 490 nm when excited with 380 nm light. The fluorescent intensity is proportional to the amount of inactive chromophore. This gives a very fast and reproducible method for quantification of small amounts of NCS (> 0.1 µg/ml).

Quantitative NCS determination by biological assay

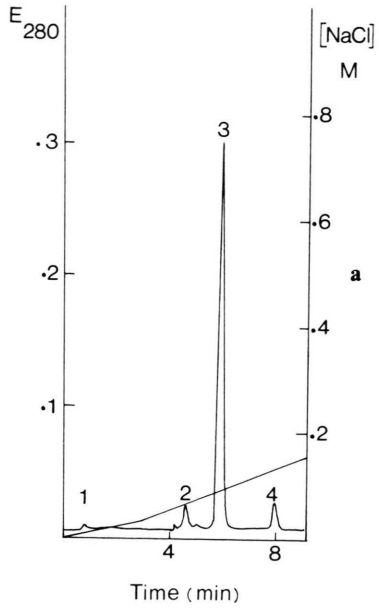
The biological activity of NCS was determined by measuring the zone of inhibition of spore germination. *B. subtilis* spores (ATCC 6633) were plated in soft nutrient agar. Samples to be tested were applied as 10 µl aliquots on 6 mm paper discs. NCS concentrations as low as 3 µg/ml could be tested. The NCS concentrations obtained from these methods agree within experimental error.

Results and Discussion

A 200 µl sample of undiluted clinical zinostatin was applied onto the Mono Q column and eluted with the described salt gradient. As can be seen from Fig. 1a practically all UV_{280}-absorbing material is bound to the column. Three well separated peaks are found in the chromatogram, mobilized at 65 mM, 90 mM, and 130 mM NaCl, respectively. Each peak was collected separately and the NCS content analysed by fluorescence and by the biological test. Only the second and the third peaks contained biological activity.

A UV irradiated sample was analysed in the same way. Fig. 1b indicates that a considerable fraction (~ 30%) is no longer bound to the Mono Q column. Furthermore peak 3 has almost disappeared while peak 4 has increased considerably. The small peak 2 is also diminished. In peak four neither a fluorescence increase nor biological activity was found. From these results it can be concluded that peak 3 in Fig. 1a represents active NCS while peak 4 in Fig. 1b is apo-NCS. This was also confirmed by measuring the absorption ratio A_{273}/A_{340} which was found to be 3.4 for peak 3 and 27 for peak 4. Next 0.5 ml of crude extract was loaded onto the Mono Q column and eluted whith the salt gradient. Biological activity was found at 90 mM NaCl. This material was diluted with salt-free buffer and rechromatographed, giving a single peak at 90 mM NaCl (Fig. 2).

Enriched crude extract was also chromatographed on the Mono Q column and the elution profile is seen in Fig. 3. Of the seven peaks collected separately the material eluting at 90 mM NaCl showed fluorescence increase and biological



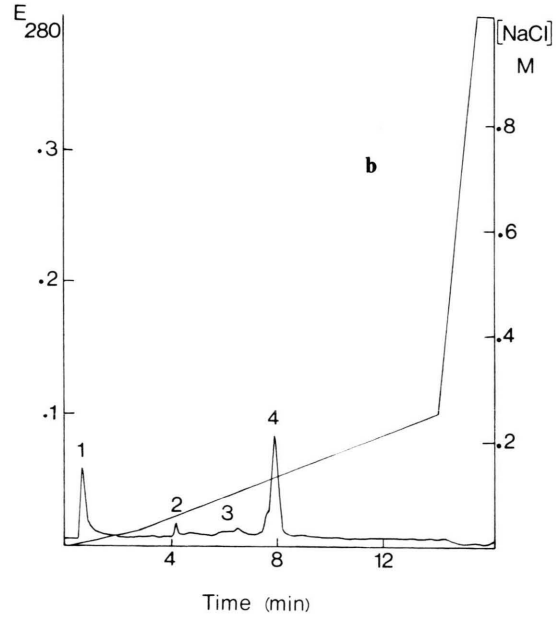


Fig. 1. Chromatogram of clinical zinostatin on a Mono Q anion exchange column. A 200 µl sample (660 µg/ml) was applied and eluted with ammonium acetate 20 mM, pH 5 by a NaCl gradient as shown; flow rate 2 ml/min; pump pressure 2 MPa; sample injection at time zero. Biological activity was found in the peaks 2 and 3. Absorption ratio A_{273}/A_{340} for peak 3 (90 mM NaCl) was 3.4, indicating native zinostatin. b. Chromatogram of 200 µl UV (335 nm) irradiated zinostatin. Surface fluence about 8 KJ/m^2. No biological activity was found. Peak 4 at 135 mM NaCl had an absorption ratio (A_{273}/A_{340}) of 27, a value characteristic for apo-NCS.

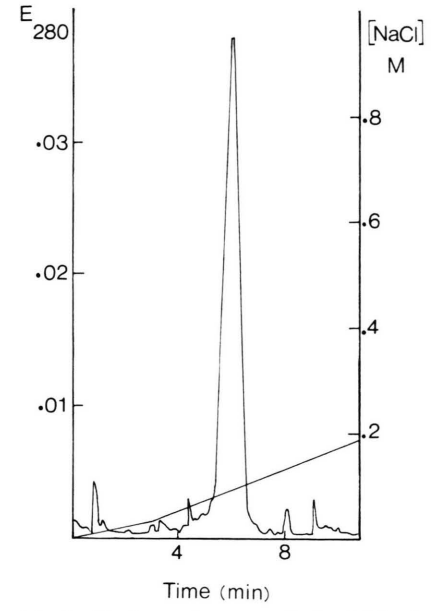


Fig. 2. Rechromatogram of the biologically active peak from a chromatogram of crude extract.

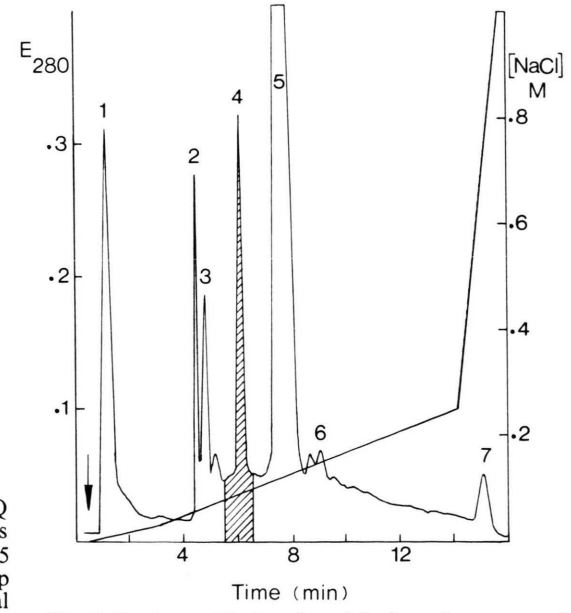


Fig. 3. Partly purified and enriched crude extract of NCS (500 µl) was chromatographed under the conditions given in the legend of Fig. 1. Arrow indicates sample injection. Of the seven separately collected peaks only peak 4 at 90 mM NaCl showed biological activity and fluorescence increase. The absorption ratio was 3.3, indicating native NCS.

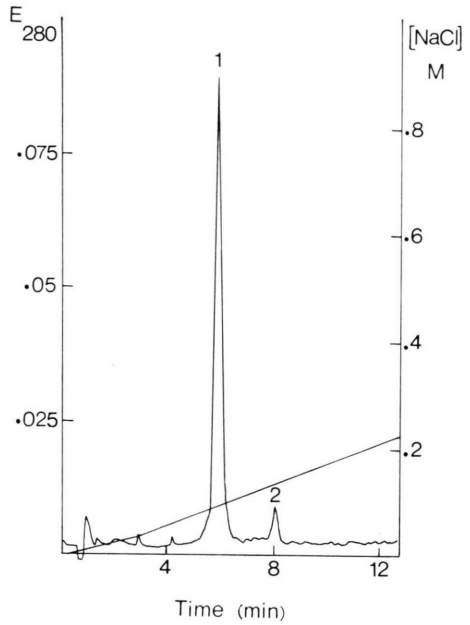


Fig. 4. Fraction 4 (cross hatched area) of Fig. 3 was diluted with salt-free buffer and rechromatographed under the same conditions as in Fig. 1.

activity. The absorption ratio A_{237}/A_{340} for this peak was 3.3, a value usually found for native NCS. Upon rechromatography of peak 4 the pattern in Fig. 4 was obtained. By comparison with the NCS peak of Fig. 1 a it can be judged that clinical grade NCS is obtained from enriched crude extract on a Mono Q column with within 20 min.

Separations were found to be very reproducible from run to run. Recovery of zinostatin activity was usually about 70%.

Conclusions

Fast protein liquid chromatography on the Mono Q anion exchange column has been successfully applied for isolation of the antitumor protein neocarzinostatin from crude extracts. The purity of the resulting preparations is comparable to that of clinical zinostatin, as can be judged from O.D. measurements and biological activity. The separation of zinostatin from apo-NCS and other contaminating impurities was achieved at low pH (5.0) and under conditions where zinostatin remains stable. Allowing 5 min for washing and equilibrating of the Mono Q column a run can be repeated after 20 min. This compares favourably with the long separation time (up to 60 h) necessary using conventional methods [18]. The high capacity of the column (25 mg protein) and a recovery of up to 70% as well the speed and reproducibility of the separations make the described system suitable for large scale preparative work.

Acknowledgements

The authors wish to thank Pharmacia Fine Chemicals AB for placing a FPLC system temporarily at their disposal. This work was partly supported by a grant from the Deutsche Forschungsgemeinschaft (Kö 347/11-2).

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