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Ampicillin polymers: identification by gel-filtration chromatography

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Summary

A method for determining the polymers formed in an aqueous solution of ampicillin is described. It is based mainly on the elucidation of molecular mass that can be assigned to the fractions separated by anion-exchange chromatography. After validation by means of a modification of the imidazole reagent, 6 peaks have been obtained. These peaks corresponds to the monomeric unit and the following 5 even polymers.

Introduction

Solutions of β-lactam antibiotics, such as penicillins and cephalosporins, form polymers on storage for a few days at room temperature. These substances are thought to be formed through a chain process involving nucleophilic attack of the amino group on the side chain on the β-lactam ring in one antibiotic molecule (Bungaard, 1976). These polymers have a great importance, since the polymerization products possess strong antigenic properties (Schwartz, 1969; Dewdney et al., 1971; Ahlstedt, 1976) as shown in animal experiments, and therefore may play a part in some clinical allergic reactions to such antibiotics.

The separation and isolation of the ampicillin polymers has been carried out by anion-exchange chromatography (Bungaard and Larsen, 1977) and by high-performance liquid chromatography (Larsen and Bungaard, 1978), while gel filtration chromatography and reversed-phase high-performance liquid chromatography have been used to analyze the polymers formed by penicillin-G (Ueno et al., 1984).

In this study we tried to isolate polymers formed in an aqueous solution of ampicillin sodium and studied their molecular mass by using chromatographic methods.

Materials and Methods

Chemicals

Ampicillin sodium (lot AS-1969) was a gift from Antibióticos S.A. (Spain), DEAE-Sephadex A-25 (anion exchanger capacity, 3.5 meq/g), Sephadex G-25 Medium, Sephadex G-15 and Blue-Dextran 2000 were purchased from Phar-

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macia (Sweden). All of the other chemicals used were of reagent grade.

Formation of ampicillin polymers

A solution (20%, w/v) of ampicillin sodium in distilled water, after adjustment of the pH to 8.5 ± 0.1 with a few drops of 2 M hydrochloric acid, was stored at room temperature for 3 days.

Ion-exchange chromatography

A column (36×2.6 cm i.d.), filled with DEAE-Sephadex A-25 gel and previously equilibrated with a 0.05 M phosphate buffer solution pH 7.2 containing 0.2 M sodium chloride, was used. One ml of the solution of polymerized ampicillin sodium was applied to the column and the polymers were eluted by increasing the salt concentration with a linear gradient from the above initial buffer to the limit one (0.5 M phosphate–1.75 M sodium chloride) (Bungaard and Larsen, 1977). The elution was carried out at a constant flow rate of 68 ml/h (12.80 ml/cm^2 · h), and column effluents were collected in 5 ml fractions. The assay was performed at room temperature.

Analytical determination of eluates

The ampicillin content in the eluates was determined according to a slightly modified imidazole method (British Pharmacopoeia, 1980): to 0.5 ml eluate were added 1 ml 0.1 M borate buffer pH 9.0 and 0.1 ml acetic anhydride. After 3 min, 2 ml imidazole reagent were added. Readings can be made after 5 min. The complex formed shows absorbance in the UV spectrum with maximum values in a range from 300 to 335 nm. So, the determination was carried out measuring the absorbance at the wavelength where the maximum was present. A UV/VIS spectrophotometer A-8531-Diode Array (Hewlett Packard, U.S.A.) was used.

In order to quantify the proportion of each polymer unit, the individual peak areas were measured with a graphic integrator (Kontron Messgerate MOP-20).

Gel filtration chromatography

An aliquot (0.5 ml) of C–F polymer fractions obtained by ion-exchange chromatography was applied separately on a column (60×0.9 cm i.d.) filled with Sephadex G-25, previously equilibrated with phosphate-buffered saline (PBS, pH 7.2, ionic strength 0.15). The elution was carried out with this buffer and the eluates were collected in 1 ml fractions. The assay was performed at room temperature.

Fractions A–C were chromatographed on Sephadex G-15 under the above conditions.

Void volume was determined with a 2% (w/v) aqueous solution of Blue-Dextran 2000.

All tests were carried out in duplicate.

Analytical determination of eluates

Elution volumes of polymers were obtained by measuring the absorbance at 220 nm. The effluent volumes corresponding to maximum concentration of the polymer (elution volume, V_e) were estimated to the nearest 1 ml from an elution diagram by extrapolating both sides of the polymer peak to an apex.

With the average elution volume and the void volume (Vo) was calculated the relative elution volume (V_e/V_O), which is related to the molecular mass of eluted polymer.

Results and Discussion

Fig. 1 shows the elution pattern of 5-ml portions of a degraded ampicillin sodium solution obtained on a DEAE-Sephadex A-25 column using a linear sodium chloride gradient at a constant pH of 7.2. This elution pattern is highly reproducible if the operative conditions are kept constant.

As can be observed, the elution profile contains 6 peaks, namely A–F although, according to this pattern, it is possible that the peak C is formed by the overlap of two or more peaks.

The peaks correspond to substances bearing the intact β-lactam ring, since the imidazole method is used to determine the β-lactam structure, and, on the other hand, their areas compared to the total area of elution profile are: peak A, 23.96%; peak B, 24.61%; peak C, 23.05% peak D, 19.77%; peak E, 5.44%; peak F, 3.18%.

After the fractionation of polymers in several peaks, the next step has been the determination of

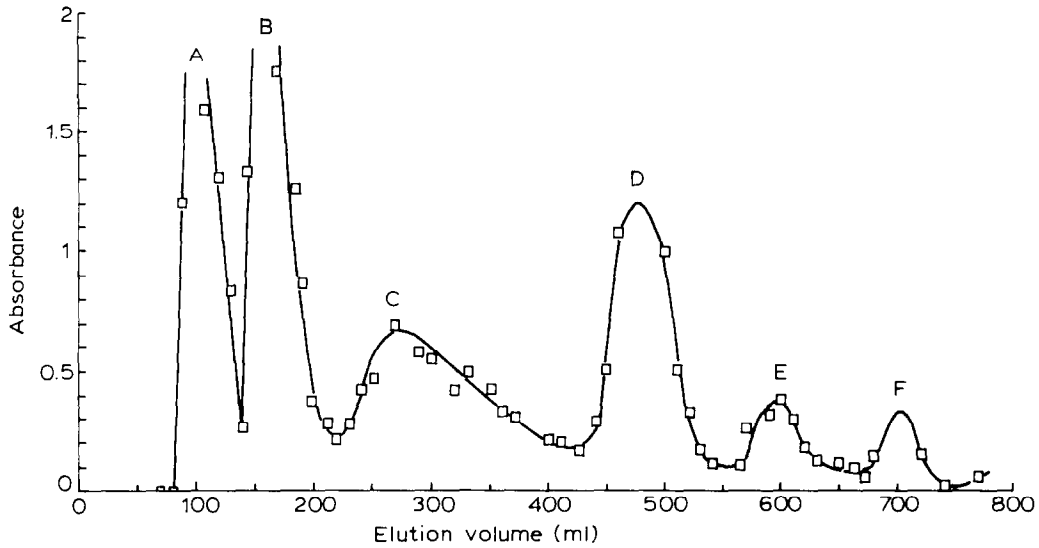

Fig. 1. Elution pattern of a 20% aqueous solution of ampicillin sodium (initial pH 8.5) kept at room temperature for 3 days. Column (36 × 2.6 cm i.d.) packed with DEAE-Sephadex A-25. Eluent, 0.05 M phosphate (pH 7.2) with a linear sodium chloride gradient. Flow-rate, 12.80 ml/cm^2 · h.

the molecular mass that may be assigned to each peak. Previously, a selectivity curve has been obtained with Sephadex G-15 by plotting the relative elution volume (V_e/V_O), variable linearly related to the elution volume, as a function of the molecular mass on a logarithmic scale. Polymers of peaks D–F have been eluted in the void volume, whereas the peaks A–C have a relative elution volume of 1.98, 1.81 and 1.41, respectively. These values imply molecular masses of 429, 613 and 1424 (Fig. 2). The assignment of 613 and 1424 values has been clear: they correspond to the dimer (M 698) and to the tetramer (M 1396); the 429 value is greater than that of ampicillin (M 349), but, at any rate, it is logical to suppose that peak A must contain the monomeric unit.

When the peaks C–F were chromatographed on Sephadex G-25, Fig. 3 was obtained. In this case, because of lack of substances with suitable molecular mass, we started from the value of tetramer (M 1396) assigned to peak C and then we supposed that peak D would be the hexamer (M 2094), peak E the octomer (M 2792), and peak F the decamer (M 3490). Plotting the logarithmic values of these 4 molecular masses vs the relative elution volume, a linear relationship was obtained (regression coefficient $r = 0.997$), which established that the proposed molecular mass fitted with the chromatographic behavior observed.

Bearing in mind the possibility of overlapping in peak C, we determined several samples at the beginning of the peak as well as at the end. Barely 0.5 ml was the greatest difference found among

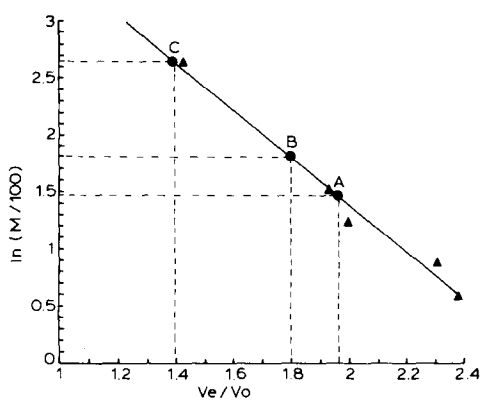

Fig. 2. Calibration line on Sephadex G-15 obtained with tyrosine (M 181), flurbiprofen (M 244), ampicillin sodium (M 349), azlocillin (M 461) and bactitracin (M 1422). Column (60 × 0.9 cm i.d.) packed with Sephadex G-15. Eluent, PBS; flow-rate, 27.72 ml/cm^2 · h.

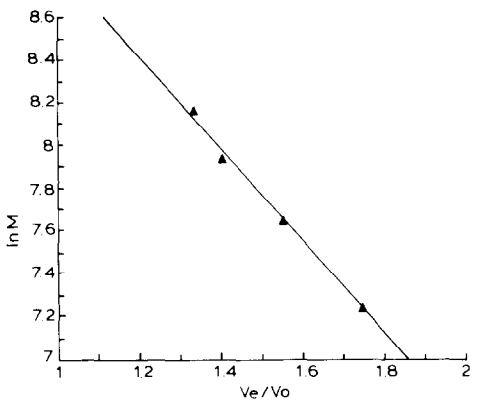

Fig. 3. Relationship between relative elution volume and molecular properties of peaks C–F on Sephadex G-25. Same operative conditions as used in Fig. 2.

the elution volumes of these samples. Hence, the peak C must be formed mainly by a single *n*-mer unit.

Based upon the above results, it can be pointed out that gel filtration is a good technique to elucidate the polymer fractions isolated by ion-exchange chromatography and that the polymers with an even degree of polymerization appear to be formed almost exclusively.

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