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POLYMERIZATION OF PENICILLINS

V. SEPARATION, IDENTIFICATION AND QUANTITATIVE DETERMINATION OF ANTIGENIC POLYMERIZATION PRODUCTS IN AMPICILLIN SODIUM PREPARATIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography with gradient elution (convex or linear gradients) on a LiChrosorb RP-8 column has been used to separate, identify and measure spectrophotometrically polymeric substances present in clinically used ampicillin sodium preparations. The method involves baseline separation of the polymerization products, ranging in size from the dimer to the octamer, both separately and from ampicillin sodium in less than 25 min with capacity factors within the range 1.5–9.9. Quantitation was effected by measurement of peak heights (absorbance at 254 nm). The minimal detectable concentrations of the products in ampicillin sodium bulk substance varied from 0.02 to 0.05% (w/w).

Analysis of three different batches of clinically used ampicillin sodium preparations by the proposed method showed the presence of di-, tetra- and hexamers in concentrations of 0.6–1.6, 0.3–2.3 and 0.1–0.4% (w/w), respectively.

INTRODUCTION

In recent years, several workers have demonstrated the formation of high-molecular-weight substances in aqueous solutions of ampicillin sodium [D-(—)-α-aminobenzylpenicillin sodium] on storage for a few days at room temperature[1–11]. These substances, so-called ampicillin polymers, are formed through a chain process involving nucleophilic attack of the side-chain amino group in one ampicillin molecule on the reactive β-lactam moiety of a second molecule[12]. The structure of such ampicillin polymers, depicted in Fig. 1, was recently conclusively established, and individual and pure, well defined polymers were obtained by anion-exchange chromatographic fractionation of a degraded ampicillin sodium solution[11].

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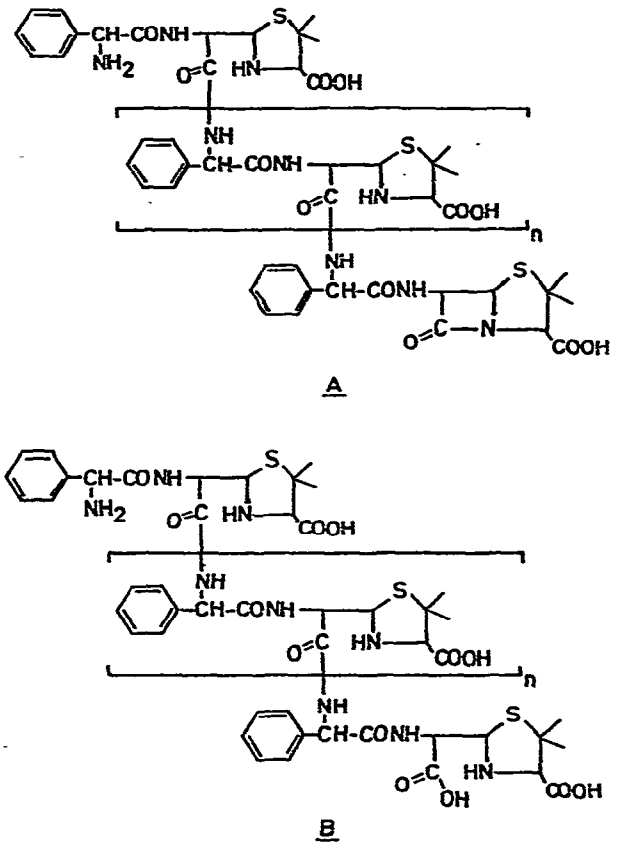

Fig. 1. Structure of the polymers of ampicillin with an intact β-lactam ring (A) or with an opened β-lactam ring in the terminal unit (B).

Polymerization products of ampicillin have been reported to possess strong antigenic properties, as shown in animal experiments[6,7,13,14], and may therefore play a part in the elicitation of some clinical allergic reactions to ampicillin. Ahlstedt *et al.*[14] demonstrated that the polymers at concentrations down to 0.1 % (w/w) in ampicillin preparations were capable of manifesting their eliciting activity. A recent clinical study by Parker and Richmond[15] indicated that the use of so-called "polymer-free" ampicillin may reduce the incidence of exanthematic adverse reactions to ampicillin preparations. In addition to their eliciting properties, ampicillin polymers have been found to be capable of inducing the formation of antibodies or sensitized lymphocytes in some experimental systems[13,16].

Because of these immunological effects of ampicillin polymers, their presence in clinically used ampicillin preparations should be controlled and kept at the lowest possible level. Unfortunately, no method has hitherto been available for the quantitative determination of such products in ampicillin bulk samples or pharmaceutical formulations. A procedure utilizing high-performance liquid chromatography (HPLC), which separates and quantifies the individual polymers (from a dimer to

an octamer) in amounts down to 0.01 % (w/w) in less than 25 min has now been developed and is described here. By means of this method, we have found substantial amounts of polymeric substances in various commercial ampicillin sodium preparations. This is the first report to show the presence of well defined polymers in ampicillin sodium bulk samples.

MATERIALS AND METHODS

Compounds

Ampicillin sodium (Doktacillin) was purchased from AB Astra, Södertälje, Sweden. Another batch of ampicillin sodium (Dumopen) was obtained from Dumex A/S, Copenhagen, Denmark. Phosphate buffer substances and acetonitrile were of analytical-reagent grade.

The di-, tetra- and hexamers of ampicillin were isolated from a 20% (w/v) aqueous solution of ampicillin sodium (pH 8.5) kept at room temperature for 3 days, as previously described[11]. Opening of the β-lactam rings in the terminal units of these substances to give products of the structure shown in Fig. 1B was effected by treatment with 0.1 *N* sodium hydroxide solution for 25 min at room temperature.

Apparatus

The high-pressure liquid chromatograph used was a Spectra-Physics Model 3500 B with accessories for programmable gradient elution chromatography and equipped with a variable-wavelength UV detector (8-μl 1-cm flow cells) and a 10-μl loop injection valve. The detector was connected to a Servogor RE 541 potentiometric recorder. The column used was a LiChrosorb RP-8 pre-packed column (Hibar) (E. Merck, Darmstadt, G.F.R.). This is a reversed-phase column (25 cm × 3 mm I.D.) containing 7-μm porous silica particles covalently linked with aliphatic hydrocarbon (C_8) groups.

Chromatography

Empirical studies showed that reversed-phase chromatography on LiChrosorb RP-8 with gradient elution was the optimal solution to the separation of ampicillin and its various polymers. The best separations were obtained with the following elution system and convex gradient elution mode.

Mobile phase A. Phosphate buffer (0.01 *M*) containing 10% (v/v) acetonitrile, pH 7.0.

Mobile phase B. Phosphate buffer (0.01 *M*) containing 20% (v/v) acetonitrile, pH 7.0.

Gradient elution. A convex gradient shape was obtained with the gradient switch of the solvent programmer module set at 6. A gradient delay time of 2 min and a sweep time of 15 min were used. The final percentage of B in A+B was set to 80. The percentage of the mobile phase B in A+B during the elution time of 2–15 min followed the equation $B\ (\%) = 80\ (t/15)^{0.5}$, where t is elapsed time in minutes.

Procedures

Analysis of ampicillin sodium bulk samples. Approximately 50 mg of ampicillin sodium were accurately weighed and dissolved in 500 μl of water. Within 5 min an

aliquot (10 μl) of this solution was injected into the column and the sample eluted at ambient temperature with the gradient elution system described above. The flow-rate was 0.8 ml/min (about 1300 p.s.i.). The column effluent was monitored at 254 nm and the UV electrometer range setting was 0.01–0.04 absorbance unit full scale (a.u.f.s.). The content of dimeric and polymeric substances in the ampicillin sodium samples was determined by comparing their peak heights with those of standards chromatographed under similar conditions.

Preparation and chromatography of external standard solutions. Solutions of ampicillin di-, tetra- and hexamer with or without an intact β-lactam ring were prepared in 0.05 *M* citrate buffer (pH 6.5) to contain from 0.1 to 10 mg/ml. An aliquot of 10 μl of each of the solutions was chromatographed to determine the purity, retention times and peak heights. The detector attenuation was varied between 0.01 and 0.1 a.u.f.s. The purity of the substances was found to be at least 90%. Graphs of peak heights *versus* amounts of substance injected within the indicated range were straight lines passing through the origin. The slopes of these standard graphs were equal (within $\pm$ 5%) to those obtained by chromatographing the compounds in admixture as well as in the presence of ampicillin sodium at a concentration of 0.1 g/ml (a correction was made for the content of polymeric impurities in the ampicillin sodium samples). The retention times for each compound were unaffected (within $\pm$ 5%) by simultaneous chromatography with ampicillin sodium of such concentration.

RESULTS AND DISCUSSION

It was found necessary to use a gradient elution system for the separation of ampicillin and its various di- and polymerization products (Fig. 1). Isocratic systems using mixtures of aqueous buffer solutions (pH 6–8) and acetonitrile or methanol resulted in incomplete resolutions or unacceptably large elution times. A convex gradient elution from 10 to 16% of acetonitrile in 0.01 *M* phosphate buffer (pH 7.0) in 15 min with a gradient delay of 2 min proved to be the optimal experimental conditions for achieving the desired separations. A linear gradient elution, using the same mobile phase systems and identical settings of delay time, sweep time and initial and final percentages of B was, however, also useful, giving only a slight increase in the time of analysis. Fig. 2 shows a chromatogram of a mixture of the various compounds as exemplified by a diluted portion of a 20% (w/v) aqueous solution of ampicillin sodium (initial pH 8.5) that had been stored at room temperature for 3 days.

Under such conditions, ampicillin undergoes a self-aminolytic degradation to produce a dimer, followed by the formation of polymers of higher molecular weight (up to an octamer)[11]. As observed earlier[11], polymers with an even degree of polymerization (dimer, tetramer, etc.) appear to be formed to a much larger extent than polymers composed of an uneven number of monomeric units (see Fig. 2). The peaks in Fig. 2 were identified by comparing their retention times with those of authentic samples of ampicillin di- and polymers, obtained by preparative anion-exchange chromatography as described in a previous paper[11], and with the retention times of the di- and polymers in which the β-lactam rings in the terminal units were opened hydrolytically. As can be seen in Fig. 2 and by the retention data in Table I, the various di- and polymers with or without an intact β-lactam ring in the terminal units are well resolved both individually and from ampicillin. With a flow-rate of 0.8 ml/min,

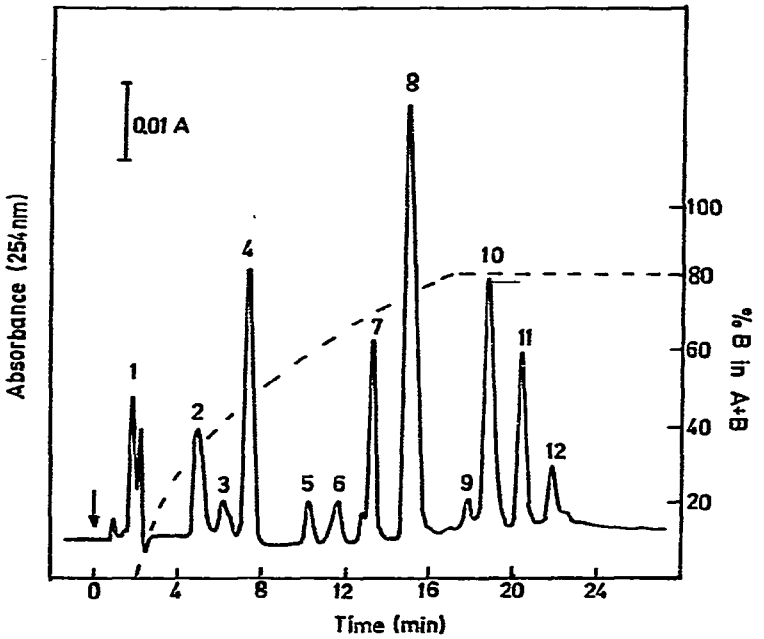

Fig. 2. Results of HPLC of a 20% (w/v) aqueous solution of ampicillin sodium (initial pH 8.5) kept at room temperature for 3 days. A 10-μl sample of a 20-fold dilution of this solution was chromatographed. A LiChrosorb RP-8 (d_p = 7 μm) column, 250 × 3 mm I.D., was used with the phosphate buffer–acetonitrile convex gradient system (– – –) described under *Procedures*. Flow-rate, 0.8 ml/min. Temperature, 22°. Detection, UV at 254 nm, 0.1 a.u.f.s. Peak identities: 1 = solvent peaks; 2 = dimer (n=0, Fig. 1B); 3 = unknown; 4 = ampicillin; 5 = unknown; 6 = tetramer (n=2, Fig. 1B); 7 = hexamer (n=4, Fig. 1B); 8 = dimer (n=0, Fig. 1A); 9 = unknown; 10 = tetramer (n=2, Fig. 1A); 11 = hexamer (n=4, Fig. 1A); 12 = octamer (n=6, Fig. 1A).

TABLE I

CHROMATOGRAPHIC PARAMETERS AND MINIMAL DETECTABLE CONCENTRATIONS OF POLYMERIC CONTAMINANTS OF AMPICILLIN SODIUM BY HPLC

Conditions as shown in Fig. 2.

Substance	*Retention time (min)*	*Capacity factor, k'*	*Minimum detectable concentration (%, w/w)**
Ampicillin	7.4	2.7	–
Dimer (n = 0, Fig. 1A)	15.0	6.5	0.02
Dimer (n = 0, Fig. 1B)	5.0	1.5	0.02
Tetramer (n = 2, Fig. 1A)	19.0	8.5	0.04
Tetramer (n = 2, Fig. 1B)	11.8	4.9	0.04
Hexamer (n = 4, Fig. 1A)	20.5	9.3	0.05
Hexamer (n = 4, Fig. 1B)	13.4	5.7	0.04
Octamer (n = 6, Fig. 1A)	21.8	9.9	n.d.

* Determined from peak heights which are at least three times the baseline noise at an attenuation of 0.01 a.u.f.s. Sample injected: 1 mg of ampicillin sodium. With UV detection at 240 nm the sensitivity is increased 2–3-fold.

separation and quantitation of the compounds are accomplished in less than 25 min. With a flow-rate of 1.6 ml/min (and a gradient delay time and sweep time of 1 and 8 min, respectively), the time of analysis could be reduced to less than 15 min without a serious loss in resolution and quantification.

The advantage of the reversed-phase system is a rapid reconditioning of the column to the initial conditions, which permits a new gradient cycle and analysis to be started within 10 min.

The retention times of the polymers increase uniformly with increasing degree of polymerization within the two types of structures (opened or closed β-lactam rings in the terminal units), and this behaviour may greatly help in the identification of peaks when no standards are available. On this basis, the peak numbered 12 in Fig. 2 is probably an octamer ($n = 6$, Fig. 1A). The formation of an octamer during storage of the ampicillin sodium solution for 3 days has been shown previously[11].

With the given elution system, the product of hydrolysis of ampicillin, α-aminobenzylpenicilloic acid[11], is not retained on the column. The product can be quantitatively determined by dissolving the ampicillin sample to be analysed in mobile phase A.

At least 1 mg of ampicillin sodium could be chromatographed without causing overloading of the column or producing interference in the di- and polymer analyses. Fig. 3 is a chromatogram of a 1-mg sample of a clinically used ampicillin sodium preparation and shows the presence of substantial amounts of dimeric and tetrameric impurities in addition to a hexamer and an unidentified substance. The results of the quantitative analysis of polymeric contaminants of this ampicillin sodium preparation and of other batches of clinically used preparations are given in Table II.

As ampicillin undergoes a rapid di- and polymerization in aqueous solution, an important precaution in the analysis of polymeric substances is to inject the dissolved ampicillin sample within at least 5 min after the dissolution. When a 10% (w/v) aqueous solution of ampicillin sodium was kept at room temperature for 5 min before chromatography, its content of dimer increased from 1.6 to 1.9% (based on the mass of ampicillin sodium). The formation of higher polymers during this period was insignificant. The possibility of the formation of di- and polymers during the chromatography can be excluded. By chromatographing the ampicillin sodium samples as 2, 5 and 10% (w/v) solutions, exactly the same content of the polymeric impurities was found.

The minimum detectable concentrations of ampicillin di- and polymers when a sample size of 1 mg of ampicillin sodium is injected into the chromatographic system are listed in Table I. These concentrations are well below the levels found for the commercial ampicillin sodium preparations analysed. The sensitivity can be increased 2–3-fold by monitoring the effluent at 240 nm instead of at 254 nm. Also at 240 nm the tendency of the gradient profile to contribute to background drift was very low.

To evaluate the precision of the HPLC procedure, 10 determinations were made on the same ampicillin sodium sample. The relative standard deviations were less than 2% for all of the contained impurities. The repeatability of the retention times for the various polymers was also estimated. The relative standard deviation was less than 1% in all instances over a period of 3 weeks.

In addition to being a valuable technique for the identification and quantitation of low concentrations of polymeric contaminants of ampicillin preparations,

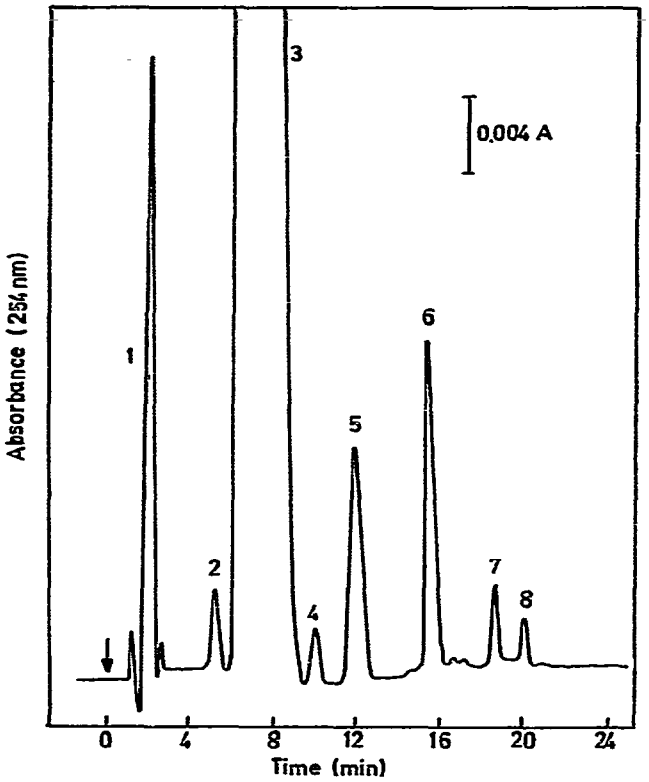

Fig. 3. Results of HPLC of a commercial ampicillin sodium sample (1 mg injected) showing the presence of various contaminants. The chromatographic conditions were identical with those in Fig. 2. The detector attenuation was set to 0.04 a.u.f.s. Peak identities: 1 = solvent peaks; 2 = dimer (n=0, Fig. 1B); 3 = ampicillin; 4 = unknown; 5 = tetramer (n=2, Fig. 1B); 6 = dimer (n=0, Fig. 1A); 7 = tetramer (n=2, Fig. 1A); 8 = hexamer (n=4, Fig. 1A).

TABLE II

RESULTS OF ANALYSIS FOR DI- AND POLYMERIC IMPURITIES IN DIFFERENT AMPICILLIN SODIUM SAMPLES CLINICALLY USED IN DENMARK

Impurity	*Ampicillin sodium sample (%, w/w)*		
	A	*B**	*C**
Dimer (n = 0, Fig. 1A)	1.0	1.6	1.4
Dimer (n = 0, Fig. 1B)	1.3	0.6	0.3
Tetramer (n = 2, Fig. 1A)	1.7	0.9	0.4
Tetramer (n = 2, Fig. 1B)	0.3	1.8	2.3
Hexamer (n = 3, Fig. 1A)	0.1	0.2	0.4

* Samples B and C were from the same manufacturer.

this HPLC method can be used for the quantitative determination of ampicillin and its various salts in the presence of hydrolysis and polymerization products. Using this method, studies are in progress for determining the time course of the formation of di- and polymers in aqueous solutions of ampicillin sodium as a function of various

variables, such as ampicillin concentration, pH and temperature. Investigations to determine whether the method as such, or in a modified form, can be extended to the analysis of di- and polymerization products of other aminopenicillins such as amoxycillin[17] and epicillin[18] are also under way.

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