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An Alternative Methodology for Determination of Cephamycin C from Fermentation Broth

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Abstract

Cephamycin C is a β-lactam antibiotic used as a raw material in several commercial antibiotics. The production and purification process of this antibiotic requires a fast, simple and accurate method to quantify it. In this paper, it was developed a high-performance liquid chromatography method to determine cephamycin C concentration, in order to offer an option for the traditional, but laborious, bioassay method normally employed. A method to obtain a calibration curve using the bioassay and cephalosporin C as standard was also proposed. The method showed more efficiency in determining cephamycin C than the bioassay, and it was simpler and faster to execute.

Keywords: Cephamycin C; Bioassay method; Quantification; Chromatography; Mass spectrometry

Introduction

Cephamycin C is a β-lactam antibiotic used as a raw material in the production of several commercial antibiotics, such as cefoxitin, cefmetazole, temocillin and cefotetan [1]. It is normally produced in submerged fermentation from cultures of *Nocardia lactamdurans* and *Streptomyces clavuligerus*. There are a couple of reports in the literature dealing with cephamycin C production by these bacteria. Batch operations using immobilized cells of *S. clavuligerus* [2], solid state fermentation [3,4], and search for alternative carbon and nitrogen sources have been reported [5,6]. Currently, cephamycin C concentrations are determined by laborious and time-consuming biological methods, which do not provide good results. Therefore, it is important to have a rapid and precise method to determine the concentration of this antibiotic in the cephamycin C production and purification processes.

The importance of β-lactam antibiotics is reflected by the numerous reports in the literature about the analysis of these antibiotics by high-performance liquid chromatography (HPLC). Rogers et al. [7] describe a method for the determination of several β-lactam antibiotics, including cephalosporin C, penicillin N, and cephamycin C, using derivatization with o-phthaldialdehyde (OPA) and detecting the peaks with a fluorescence-measuring device. Based on their method, it was possible to design a highly sensitive method with high resolution to determine these antibiotics in fermentation broths.

In another paper, Rogers et al. [8] describe the need for derivatization of β-lactam antibiotics to increase their detection sensitivity. In the case of penicillins, the authors describe the use of a solution containing imidazole and mercury chloride (pre-column derivatization), and ultraviolet determination. The authors also describe another method using post-column derivatization and reaction of the antibiotic with OPA, for the analysis of cephalosporins and penicillins. Both methods yield high-resolution chromatograms and can be used to determine β-lactam compounds in fermentation broths.

Holzhauer-Rieger et al. [9] describe an on-line method for the determination of cephalosporin C, penicillin N and other antibiotics during the culturing of *Cephalosporium acremonium*. The method does not require derivatization and cephalosporin C is determined with high precision at high-resolution peaks.

In a patent filled by Kamogashira et al. [10], the authors describe the determination of cephamycin C concentrations without derivatization of the antibiotic, using an acetic acid solution as the mobile phase and a Waters C-18 μ-bondapak column. The chromatogram and the cephamycin C peak obtained using this chromatographic method was not described in the patent.

Another method for determining cephamycin C without derivatization is reported by Leitão et al. [11]. The method involves the use of phosphate buffer as the mobile phase and a Waters C-18 μ-bondapak column. However, in the chromatogram obtained using this method, the cephamycin C's peak was poorly resolved.

In a specified literature about quantification of metabolites produced by *Streptomyces clavuligerus*, Liras and Martín [12] describe the use of a method for cephamycin C determination by HPLC without derivatization. The methodology proposed was able to separate some intermediates in the metabolic pathway of cephamycin C, but the resolution of the chromatographic peaks obtained was low. Therefore, determination of cephamycin C concentration by this methodology did not provide high precision.

Although there are numerous methods that use HPLC for cephamycin C determination, these analytical methods are difficult to implement due to the lack of a commercial standard. Therefore, biological assays for the determination of this antibiotic, such as that described by Liras and Martín [12], are still widely used. The authors argue that penicillin N has no influence on the determination of cephamycin C concentration in fermentation broths from wild microorganisms. They describe the use of cephalosporin C as standard

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and the difficulties caused by the absence of cephamycin C as standard.

As can be seen, cephamycin C determination by a biological method is limited not only due to the lack of precision of the measurements, but also because they are laborious and time-consuming, which greatly hampers their production and purification processes.

Batista Neto et al. [13] described a methodology for purification of cephamycin C from fermentation of *Streptomyces clavuligerus*. The authors used membrane filtration processes - microfiltration and ultrafiltration - followed by chromatographic steps with nonspecific and anionic resins. The results obtained showed the efficiency of the purification methodology proposed.

In this context, this paper proposes a new HPLC method for cephamycin C determination in fermentation broths without the use of a standard. UV spectrum and high-resolution mass spectroscopy were used to indentify cephamycin C in an isolated chromatogram peak. To quantify cephamycin C concentration, experimental data obtained by bioassay were correlated with the peaks areas from the UV chromatogram for cephamycin C.

Materials and Methods

Materials

HPLC grade water and acetic acid were used in HPLC runs and in LC-MS analysis. Water was prepared using a Milli-Q system at a resistivity of 18 MΩ.cm (Millipore, USA). Acetic acid was purchased from Mallinckrodt (USA). The resins used in broth treatments were Amberlite XAD 1180 and Q Sepharose XL, purchased from Sigma (France) and GE (Sweden), respectively. To determine cephamycin C concentration by bioassay the following reagents were used: Cephalosporin C (Sigma, USA) as standard, agar (Acumedia, USA), beef extract (Acumedia, USA) and peptone (Acumedia, USA). All the others reagents were analytical grade.

Fermentation broths

The tests were carried out using fermentation broths from batch cultivations of *S. clavuligerus* for cephamycin C production. The culture media used for this fermentation contained, as main constituents, glycerol or starch as carbon source, and hydrolysed soybean protein or cottonseed flour as nitrogen source [6]. The samples were obtained as follows:

• Raw Broth (RB): samples were obtained by filtering the fermentation broth through a 0.22 μm pore-size filter.

• Treated Broth (TB): samples were obtained by filtering the raw broth through 3 kDa membranes to remove high-molecular weight proteins. The filtered material was injected onto a column containing XAD 1180 resin for removal of contaminants. After that, the resulting broth was injected onto a column packed with the resin Q Sepharose XL. This procedure allowed to obtain a more concentrated and purified sample of cephamycin C, that was used in the chromatographic tests.

Quantitative and qualitative determination of cephamycin C by bioassay

Cephamycin C concentration was determined by biological assay as described by Liras and Martín [12], using *Escherichia coli* ESS as the indicator microorganism. Plates were prepared with nutrient agar (5 g/l peptone, 3 g/l beef extract, 15 g/l agar, pH 7.2), seeded with *E. coli* ESS. As cephamycin C is not commercially available, cephalosporin C was used as reference antibiotic. The amount of cephamycin C was defined in terms of units of cephalosporin C (U_{CephC}), where 1 U_{CephC} corresponds to the amount of cephamycin C required to produce an inhibition halo equal to that produced by 1 mg of cephalosporin C.

Determination of cephamycin C concentrations in TB samples was performed in quintuplicate in order to ensure low error values, while that of the RB was performed in triplicate.

Qualitative tests were also carried out. Plates were prepared in the same way as in quantitative method. The difference in qualitative method is that only undiluted samples, without dilution, were applied in the plates. These assays were used only to check the presence of antibacterial activity of samples and collected fractions. Inhibition zones formed in agar indicated the presence of a compound with antibacterial activity.

HPLC analysis

Chromatographic analyses were carried out in a Waters HPLC system (Waters, USA) equipped with two pumps, temperature control, automatic injector and 996 PDA detector. The first chromatographic condition was based on the method described by Kamogashira et al. [10]. The mobile phase used was a 0.01M solution of acetic acid, and the temperature of the column was maintained at 28°C. Peaks were detected at a wavelength of 254 nm. UV scan spectra were also obtained for the peaks supposed to be cephamycin C. Four methodologies were tested. The differences between them, described below, were the stationary phase and the flow rate of mobile phase.

• **Method 1:** C-18 μ-Bondapak (Waters, Ireland) (10 μm, 3.9 mm i.d. x 300) - 2 $mL.min^{-1}$

• **Method 2:** C-18 μ-Bondapak (Waters, Ireland) (10 μm, 3.9 mm i.d. x 300) - 1 $mL.min^{-1}$

• **Method 3:** Synergi MAX RP-12 (Phenomenex, USA) (4 μm, 4.6 mm i.d. x 250) - 1 $mL.min^{-1}$

• **Method 4:** Synergi MAX RP-12 (Phenomenex, USA) (4 μm, 4.6 mm i.d. x 250) - 2 $mL.min^{-1}$

LC/MS analysis

High-resolution mass spectra were obtained by liquid chromatography-mass spectrometry, applying electrospray ionization in positive mode (ESI (+)). The mass spectrometer used was a Bruker Daltonic Micro TOFF (Germany). These analyses were performed at the analytical centre of the Chemistry Institute from University of São Paulo, in Brazil [14].

Results

The tests carried out with Method 1 did not yield satisfactory results. The chromatogram in Figure 1A shows the results obtained when Method 2 was used. During this chromatographic run, fractions were collected at regular time intervals and then these fractions were submitted to qualitative bioassay tests to check antibacterial activity. Fraction 6 presented antibacterial activity, therefore the scan UV spectrum corresponding to the peak at retention time of 6.4 min was extracted (Figure 1B). A comparison made with the scan UV spectrum of cephamycin C described by Kamogashira et al. [10] suggested that the peak at retention time of 6.4 min was a strong candidate to be cephamycin C.

Methods 3 and 4 were carried out in order to test selectivity and band spacing in a C-12 column. As it is shown by Figure 2, this column provided better band spacing than the C-18 column. The presumed

peak of cephamycin C was better separated from the other peaks than it was when method 2 was used, and cephamycin C was also more retained by the stationary phase. Nevertheless, as method 3 used a smaller flow rate, it consisted of a long time run for each chromatogram. The presumed peak of cephamycin C presented a retention time of 21.6 min. To reduce the chromatographic run time, in method 4 the flow rate of the mobile phase was doubled, which enabled to obtain a chromatogram with a shorter run time. Methods 3 and 4 resulted in similar chromatograms. Figure 2A shows the chromatogram obtained with method 4, while Figure 2B shows the scan UV spectrum of the presumed chromatographic peak (9.5 min) of cephamycin C.

After obtaining an assumed peak of cephamycin C at high resolution, the sample was injected, according to method 4, into the LC-MS system. This procedure resulted in the UV and ionization chromatograms illustrated in Figure 3A and 3B, respectively.

The Figure 4 shows the mass spectrum of the chromatographic peak at a retention time of 9.7 min. The ratio *m/z* obtained for this peak was 447, 1189, which is practically equal to the exact mass of the cephamycin C molecule plus one hydrogen atom. This value is in concordance with the literature, which presents the exact mass of cephamycin C equal to 446.110749 [15].

This finding allows concluding that the compound appearing at 9.7 min is cephamycin C and that the concentration of this β-lactam antibiotic can be determined successfully by the proposed method.

After determining the peak of cephamycin C, the calibration curve was drawn using five samples of treated broth (TB). Concentration of total cephamycin C in these samples were determined by bioassay, performed in quintuplicate. Four different volumes of each sample were injected into the HPLC system using the method just developed. The peak area obtained by the HPLC analysis was plotted against the concentration obtained by the bioassay (Figure 5A). The following calibration equation relating the chromatographic peak area, in arbitrary units (AU), to the amount of cephamycin C, in U_{CephC}, was obtained:

$$\text{Peak Area (AU)} = 2{,}977 + 2.5\text{x}108\ U_{CephC} \quad (1)$$

The value of the correlation coefficient ($R^2 = 0.98$) and the graphic

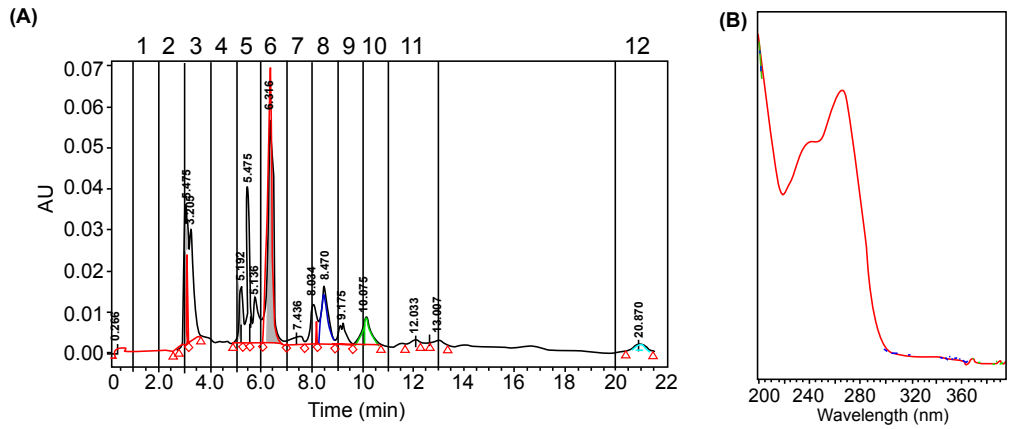

Figure 1: (A) Chromatogram of a sample of TB obtained by Method 2 (λ=254 nm), and (B) UV scan spectra corresponding to the peaks at retention time of 6.4 minutes.

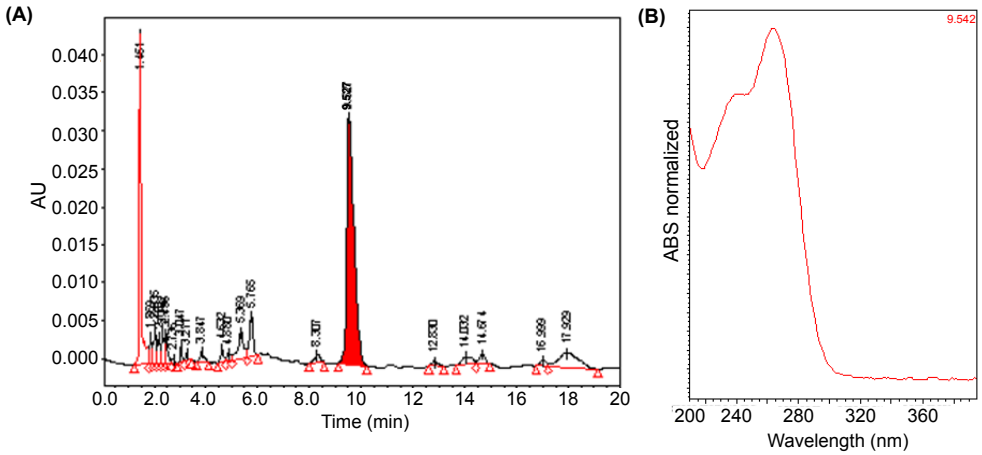

Figure 2: (A) Chromatogram of a sample of TB obtained by Method 4 (λ = 254 nm), and (B) UV scan spectrum of corresponding to the peak at 9.5 minutes (presumed to be the peak of Cephamycin C).

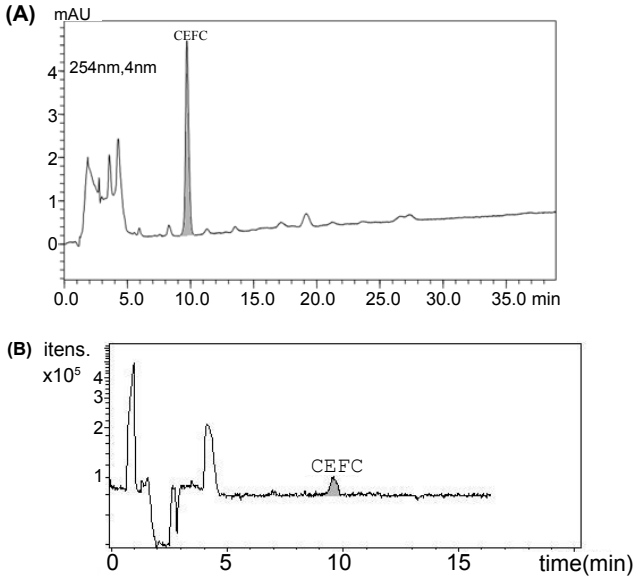

Figure 3: UV (A) and Ionization (B) chromatograms obtained at λ = 254 nm in LC-MS.

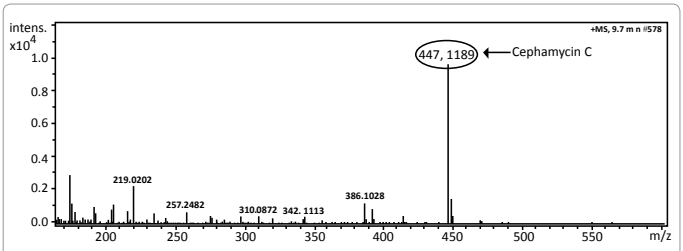

Figure 4: Mass spectrum of the peak at a retention time of 9.7 minutes (Method 4).

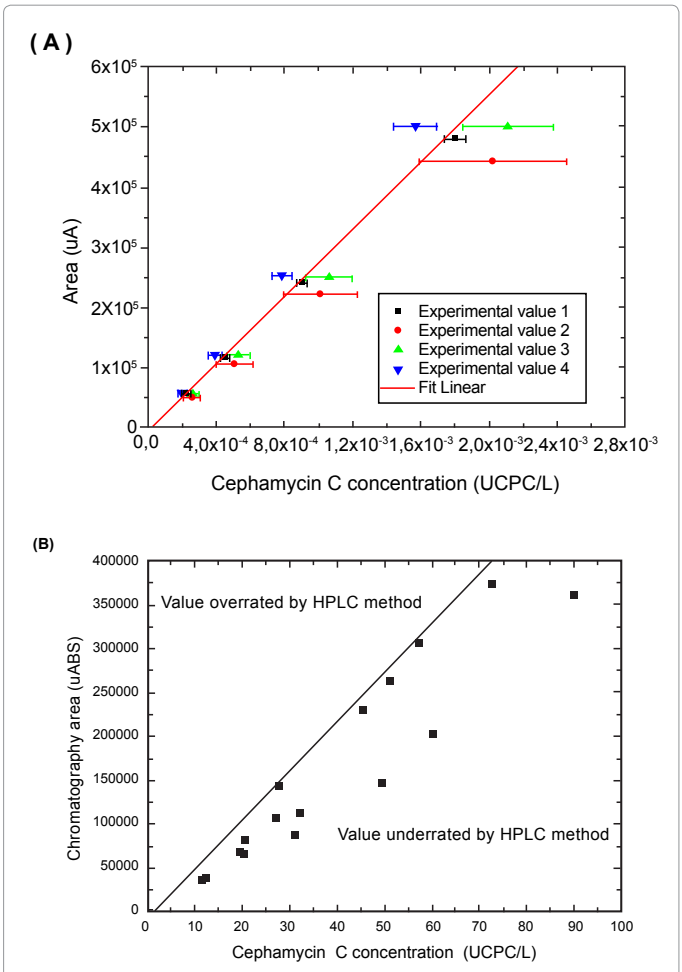

Figure 5: (A) Calibration curve for quantification of Cephamycin C in UCephamycin C, and (B) comparison between Cephamycin C values determined by the proposed method (line) and those obtained by bioassay (dots).

of Figure 5A show the good fitting of the calibration equation to the experimental data.

A comparison between the HPLC and bioassay methods was made, by determining cephamycin C concentration in raw broth samples using the two methods (Figure 5B). The results showed that the experimental values determined by the biological method (dots) were all equal to or higher than the values estimated by the proposed method (line). The chromatographic method could overestimate the mass of this antibiotic, if there were some compound with the same retention time of cephamycin C, but it could not underestimate it. The results from bioassay indicate the interference of some product produced by the microorganism, different from the cephamycin C, which presents biological activity detected by the bioassay. Therefore, Figure 5B shows that the results obtained by the chromatographic method are more accurate than those obtained by bioassay.

Studies on the purification of cephamycin C demonstrated the presence of other compound (or compounds), besides cephamycin C, in fermentation broth and that are detected by the bioassay method [13]. Rollins et al. [16] reported the production of β-lactam compounds by the biosynthetic pathway of cephamycin C production in conditions of dissolved oxygen limitation. Some of these compounds, like penicillin N, present antibacterial activity that may interfere in the determination of the cephamycin C concentration by biological methods. Other HPLC methods, such as described by Rogers et al. [7] and Rogers et al. [8], described the determination of cephalosporins, including cephamycin C, using derivatization with o-phthaldialdehyde (OPA). The proposed method is simpler because it does not require sample derivatization.

The results showed that the bioassay method may overestimate the concentration values of cephamycin C. Therefore, the HPLC method provides much better results, i.e., more accurate measures than those obtained by the method currently in use (bioassay), enabling several studies on the production and purification of cephamycin C to be carried out.

The results obtained here confirm that an efficient method with high chromatographic resolution was successfully developed and implemented for the determination of cephamycin C by HPLC. Based on the mass spectrum, it was possible to determine the retention time of cephamycin C and the peak to which it corresponds in a high resolution chromatogram. It was also possible to obtain a calibration curve. The proposed method provides much better results than the bioassay method, proving its greater efficiency and yielding faster and more accurate results in studies on the production and separation of this antibiotic.

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