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Determination of ceftazidime concentration in Mueller Hinton agar by high-performance liquid chromatography

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Abstract

A simple and rapid RP-HPLC method for the direct determination of ceftazidime (a β-lactam antibiotic) in agar (Mueller Hinton Agar-II) was developed. The method, characterised by good precision (C.V.$\leq$7.9%) and linearity in the 5–200 μg/ml range ($r^2 \geq 0.998$), showed high recovery from agar (104$\pm$8%) and a sensitivity limit of 1.4 μg/ml. The analytical procedure allowed the determination of the "true" antibiotic concentration in the agar matrix. In addition, the small volume sample may allow a precise evaluation of the antibiotic levels point by point in the agar plates necessary to study the kinetics of diffusion of ceftazidime.

Keywords: Mueller Hinton agar; Ceftazidime; Antibiotics

1. Introduction

The agar diffusion susceptibility disk test (Kirby–Bauer) is one of the most utilised assays in clinical practice to determine the susceptibility of a microorganism to antibiotics [1]. The assay is performed by applying onto an inoculated agar plate a dried standardised paper disk containing a definite amount of antibiotic. The susceptibility of the organism tested is proportional to the diameter of the growth inhibition zone around the disk, and the minimum antibiotic concentration that inhibits the bacterial growth (MIC = minimum inhibitory concentration) is lower the larger the diameter of the inhibition zone [2].

One of the main purposes of the microbiology laboratory is to support the clinician in the choice of the antibiotic therapy, and to allow dosage adjustment during the treatment. The exact concentration of an antibiotic at the edge of inhibition zone in agar diffusion could be quantified only by means of a method permitting the direct quantification at fixed times in fixed points of the plates. Hoette and Struyk [3] proposed a very time-consuming microbiological procedure for the quantification of antimicrobial agents diffusing in uninoculated blood agar plates. Since then, few papers on direct detection of antibiotics in agar were published, mostly with microbiological methods and focussing on the first antibiotics synthetized 20–30 years ago. Microbiological methods cannot be used in the presence of bacterial growth as is the case when we work with routine susceptibility tests. The correlation between the MIC and the diameter of the inhibition zone is

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calculated for every antibiotic/bacterial species with a regression curve between the MIC in broth and diameter of the inhibition zone in agar, without directly taking samples of the agar plates. We have found only one study of direct detection of antibiotics in agar with HPLC, which, however, does not give very exhaustive technical information [4].

The detection of antibiotics in plasma or other biological fluids by means of HPLC has been studied for most of the β-lactam antibiotics and, within this group, for the cephalosporins, a class of antibiotics largely employed in clinical practice [5–14]. Leeder et al. [15] proposed a simple method for ceftazidime determination in serum and urine by deproteinization of the samples with methanol, while Chan et al. [16] purified ceftazidime, ceftriaxone and other cephalosporins from serum by adding to the samples a solution of an internal standard (I.S.) in acetonitrile (a cephalosporin other than the one being assayed) in order to obtain simultaneous drug extraction and deproteinization of the matrix. The pre-analytical procedures for antibiotic purification from biological fluids are generally represented by deproteinization with organic solvents and direct injection onto the HPLC system [15], by double extraction with organic solvents followed by a back-extraction in aqueous phase [17], or by a preliminary purification with anion-exchange chromatography [18].

In this paper we describe a method for the measurement of a third-generation cephalosporin (ceftazidime) (CAZ) in samples extracted from Mueller Hinton Agar-II (MHA-II) plates, based on high-performance liquid chromatography (HPLC). CAZ is a semisynthetic antibiotic with enhanced in vitro activity against a wide variety of Gram-negative organisms [19], particularly *Pseudomonas aeruginosa* [20]. The method was employed to study the CAZ diffusion kinetics in agar.

2. Experimental

2.1. Chemicals

Methanol (HPLC grade) was purchased from BDH (Milan, Italy), perchloric acid (PCA), trifluoroacetic acid (TFA), trichloroacetic acid (TCA), hydrochloric acid (HCl) and potassium phosphate bi-basic (K_2HPO_4) were from Fluka (Buchs, Switzerland). All the buffers used were prepared in bi-distilled water, filtered and degassed by filtration (0.45 μm, Millipore Corp., Bedford, MA, USA).

2.2. Drug standards

Pure drug standards were obtained as follows: ceftazidime (CAZ) from Glaxo (Verona, Italy), ceftriaxone from Hoffmann-La Roche (Basel, Switzerland), cefazolin from Bristol Italiana (Latina, Italy), ampicillin and theophylline from Farmitalia-Carlo Erba (Milan, Italy). The Sensi-disc, Susceptibility Test Discs (CAZ-30 μg) were all from the same batch and purchased from Becton Dickinson (Cockeysville, MD, USA). The disks were stored at −20°C until the week of the experiment.

2.3. Agar medium

Flasks with Mueller Hinton Agar-II (MHA-II) were purchased from Becton Dickinson, all of the same batch.

2.4. Standard solutions

Ceftazidime stock solution (1 mg/ml) was prepared in bi-distilled water and aliquots were stored at −80°C. The working solutions were prepared daily by diluting the stock solution with water. Theophylline stock solution (1 mg/ml) was prepared in water, and stored at −80°C. Daily, theophylline working solution (1 ng/μl) was prepared by diluting the concentrated one with 6% PCA.

2.5. MHA-II plate preparation

Flasks with MHA-II were heated (95°C) in a boiling water bath till melting of agar. Then, 23-ml aliquots of melted agar were poured into sterilised Falcons pre-heated at 50°C and allowed to stand at this temperature for 15 min in a thermostated bath. After reaching 50°C, the medium was poured in sterilised plates (9 cm diameter) and allowed to solidify at room temperature. The resulting agar depth was 4 mm. The plates were dried in a low-traffic, dust free area to avoid contamination.

For linearity, precision and recovery studies, plates

with known and homogeneous concentrations of CAZ, were prepared by adding different amounts of ceftazidime standard solution to the melted medium thermostated at 50°C (final volume 23 ml). The Falcons were gently rotated for 15 min to allow uniform distribution of the drug into the medium, then the agar was poured into the plates and left to solidify.

2.6. Apparatus

A Kontron HPLC system (Zurich, Switzerland) consisting of two dual-piston pumps (Model 420), an autosampler (Model 460), and a double beam UV detector (Model 430), was used. The apparatus was computer controlled by a Data System (Model 450) for the collection and the elaboration of data. A reversed-phase LiChrosphere 100 RP-18 (125×4.0 mm, 5 μm) (Merck, Darmstadt, Germany) column was employed.

2.7. Sampling from agar and CAZ extraction

Two cylindrical samples (3.1 μl each) were harvested by means of a glass capillary (1.0 mm I.D.), at opposite points with respect to the centre of the plate. The distances between the points were measured with a caliper and marked on the external bottom of the plate. The two agar cylinders were put together in an Eppendorf tube and spiked with the I.S. (200 ng) in 200 μl of 6% PCA. The samples were then heated at 50°C for 15 min in a block heater, neutralised by adding solid $NaHCO_3$, centrifuged (13 000 *g*, 2 min), filtered (0.45 μm) and automatically injected onto the HPLC system (170 μl).

2.8. Chromatography

The analysis was performed in isocratic mode, eluting the column at a flow-rate of 1.5 ml/min, with a mobile phase of potassium phosphate (K_2HPO_4) 70 m*M*, pH 6.5–methanol (93:7, v/v). The separation was carried out at room temperature with monitoring at 255 nm.

2.9. Quantitative analysis

The unknown CAZ concentration in the samples was quantified on the base of aqueous and agar calibration curves in which the CAZ/theophylline peak area ratio (A/A) was plotted against the initial weighed CAZ concentration (μg/ml). Linear regression analysis was performed obtaining the parameters for the quantification.

2.10. Diffusion of CAZ in agar

After deposition of a sensitivity disk containing CAZ (30 μg) in the middle of MHA-II plates, the diffusion of CAZ was studied as a function of the time and the distance from the disk edge. Samples were taken with a glass capillary at 3 and 12 mm from the middle of the disk, at 2, 4, 6, 16, 24 h after the deposition.

3. Results

3.1. Optimisation of the chromatographic method

Starting from the data published by Leeder et al. [15], we initially performed the CAZ analysis employing KH_2PO_4 150 m*M*, pH 6.5–methanol (82:18, v/v) as mobile phase. While these conditions seemed suitable for the pure standard, they proved to be unsuitable when the drug in agar was analysed. The analytical conditions were therefore modified according to those reported in Section 2, resulting in a ceftazidime t_r of 5.7 min and a chromatographic profile free of interfering peaks (Fig. 1).

Initially were tested various antibiotics (cefazolin, ceftriaxone, ampicillin) as I.S., but all were found to be unsuitable. So, on the basis of Leeder's results with 8-chlorotheophylline [15], we tested theophylline, which showed a t_r of 16 min and no interferences due to the agar matrix.

3.2. Optimization of the pre-analytical procedure

Because of the complexity of the MHA-II composition, the extraction of ceftazidime was first approached by using the organic solvents acetonitrile, methanol and acetone. The recoveries were

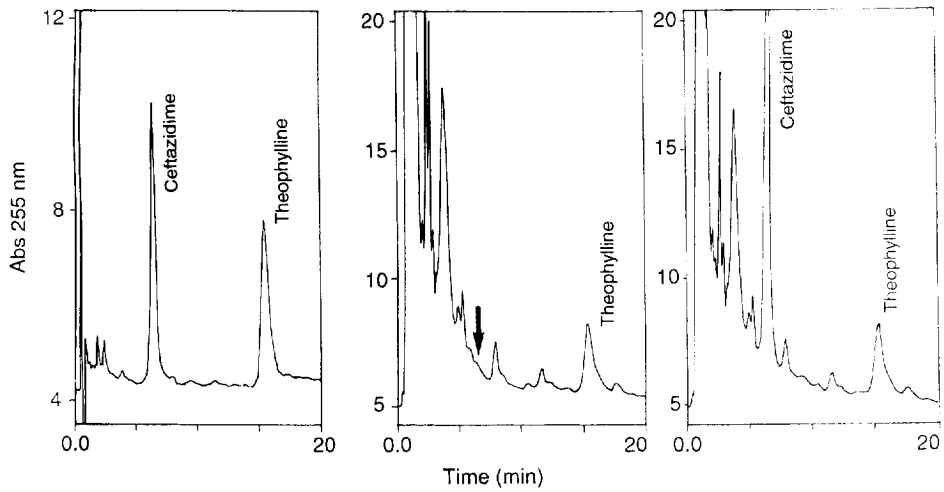

Fig. 1. Chromatographic profiles of (A) pure standards: CAZ 115 ng and theophylline (I.S.), 180 ng injected; (B) sample from MHA-II plate without CAZ (blank). I.S., 170 ng injected; (C) sample from MHA-II plate containing an homogeneous CAZ concentration of 50 μg/ml (250 ng injected), I.S., 170 ng injected. Analytical conditions as reported in Section 2.

very low and the peaks appeared large and tailed. Then, as described by Aldous et al. [4] for agar matrix, we used water at pH 4.5, resulting in very dirty extracts with numerous interfering peaks. A stronger acidic treatment was therefore performed by using 5% TFA, 6% TCA, HCl 60 m*M* or 3 *M*, and 6% PCA. The best results were obtained with 6% PCA, resulting in a chromatogram free from interferences and a recovery of about 60%.

3.3. Influence of temperature on ceftazidime stability

In order to improve the recovery of the antibiotic from the agar matrix we tried heating the samples under acidic conditions (6% PCA). We previously carried out studies on CAZ stability at high temperature by heating the standard solutions in 6% PCA for 1 h at 30, 40 or 50°C and treating as described for agar samples. The peak areas obtained were compared with those of the untreated standards. Recovery of the drug was always quantitative and no other peaks due to possible degradation products appeared in the chromatogram. After heating at 50°C for 15 min, the CAZ recovery rose to 98±2%.

3.4. Linearity and recovery from agar

Linearity was tested on CAZ standard in water and agar. Aqueous standard curves ($n=6$) in the 5–200 μg/ml range were set up by diluting the stock solution of CAZ (1 mg/ml), adding the I.S. (400 μl=400 ng) and processing the samples as described before. Plotting the area ratio CAZ/I.S. vs. the CAZ concentration, the linear regression analysis gave a mean slope of 0.0515±0.00057 (mean±S.D.), an intercept of −0.0085±0.071 and $r^2 \geq 0.998$.

The linearity of the method in agar was assessed by analysing samples extracted from MHA-II plates ($n=8$) prepared with a known and homogeneous concentration of CAZ in the 5–195 μg/ml range, obtaining a mean slope of 0.0475±0.0017, an intercept of −0.01975±0.06 and $r^2 \geq 0.998$.

Recovery was determined in the 5–195 μg/ml range by comparison with untreated aqueous standard. The mean recovery (mean±S.D., $n=12$) was 104±8.0%.

3.5. Precision

The intra-run precision in agar was calculated analysing plates at known concentration (10, 20, 97.1

ng/μl). The mean C.V.% ($n=10$) were 7.9, 4.4, 2.4% respectively. For between-day precision, the same plates were re-analysed for 4 days consecutively giving an inter-assay C.V.% always below 7% ($n=4$) for each tested concentration.

3.6. Sensitivity of method

Assuming a signal-to-noise ratio of 3, the sensitivity of the assay was estimated to be 10 ng injected onto the HPLC system, this level corresponding to 1.4 μg/ml in agar medium.

3.7. Ceftazidime diffusion in agar

The sampling points in the agar plates were chosen at 3 and 12 mm from the disk edge. In Fig. 2 the curves obtained from the experiments are reported, evidencing that at 3 mm CAZ reached its maximum concentration after 6 h of incubation, while at 12 mm from the edge of the disk the antibiotic levels were undetectable in the first 4 h, reaching the highest concentrations in the 16–24 h interval.

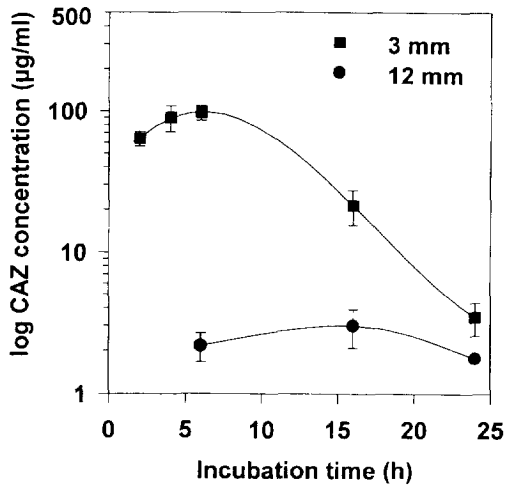

Fig. 2. Diffusion of CAZ in agar. CAZ concentration measured at 3 mm and 12 mm from the disk center. The values are expressed as mean ($n=8$) ± S.D. The concentrations measured at 12 mm at 2 and 4 h after incubation were undetectable.

4. Discussion

In the last 30 years very few studies have been carried out on the diffusion of antimicrobial agents in agar because accurate analytical procedures were not available. The time-consuming and imprecise microbiological method previously described by Hoette and Struyk [3] allowed one to study the kinetics of diffusion of penicillin, streptomycin, chloramphenicol and oxytetracycline only in uninoculated plates and, therefore, was not representative of an antibiogram test.

Over the last 10 years the determination of antibiotic concentration in body fluids has been more and more frequently performed by means of chromatographic methods coupled with various pre-purification procedures [16–23]. The pre-analytical purification set up developed in our laboratory allows in one single step the quantitative extraction of the drug, the deproteinization of the agar matrix and the spiking of the internal standard.

During their studies, McCormick et al. [6] and Signs et al. [7] described the degradation of cephalosporin molecules caused by extreme pH conditions, suggesting the possible formation of the lactone form. In our extraction conditions (6% PCA, 50°C for 15 min) we did not note loss or degradation of CAZ while, utilising organic solvents as described by Uihlein et al. [22] we always obtained a low recovery and the simultaneous extraction of interfering substances. Aldous et al. [4] studied the extraction and HPLC analysis of micro-samples (about 15 μl) containing cefotaxime or cefamandole from agar plates. Unfortunately neither the medium employed nor the chromatographic conditions were reported and no chromatograms were shown.

The chromatographic procedure reported here for agar samples is based on the determination of cephalosporins in biological fluids. Chan et al. [16] carried out the simultaneous analysis of nine cephalosporins (ceftazidime was included) using a mobile phase containing an ion-pairing reagent. The chromatographic analysis set up by Brisson and Fourtillan [17], utilising acetate buffer and methanol, was rather simple, but the pre-analytical procedure was performed with a solvent extraction followed by an aqueous re-extraction.

Our aim was to develop a rapid, precise and

sensitive method, not requiring dedicated instrumentation. The isocratic analysis allowed the determination of ceftazidime in about 16 min, avoiding the long column equilibration time, necessary in the case of analysis employing pairing-ions or organic solvent gradient. The choice of the 255 nm wavelength was made on the base of the absorbance spectra of ceftazidime and theophyilline. The sensitivity limit (about 1 ng/μl) was below the CAZ concentrations found at the inhibition zone edge reported by NCCLS [23]. Our method, carried out on small sample volumes, allowed a precise evaluation of the antibiotic levels at any point in the agar plates and thus the study of the kinetics of diffusion of the antibiotic in agar. The acid extraction procedure and the subsequent filtration step also made it possible to quantify the drug in samples extracted from plates with bacterial growth as in routine clinical tests. The HPLC method proposed is also characterised by short analysis time: $\leq$48 h are required to extract, purify and analyse 90 samples resulting from a diffusion kinetics study performed in triplicate, including interpretation of data.

The determination of CAZ by HPLC allows the measurement of the antibiotic concentration in the real conditions of an antibiogram test and will be useful to get a better understanding of the critical points involved in the formation of the inhibition zones and to analyse the interaction between drug and microorganism.

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