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Is the direct quantitation of antibiotics in agar by high-performance liquid chromatography useful?

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Abstract

The direct quantification of antibiotics in agar allows one to study the quality of the agar matrix, the kinetics of diffusion and the bacteria–antibiotic interaction. Mueller–Hinton agar (MHA) plates from three manufacturers were tested using HPLC and the disc diffusion test of ceftazidime (CAZ). Notable differences in the chromatographic profiles of MHA plate extracts from OXOID, DID and Becton Dickinson (BD) were shown, with a higher CAZ concentration after 24 h at 6 mm in BD *P. aeruginosa* inoculated plates (5.1±1.7 μg/ml, $n=6$) vs. OXOID and DID (1.6±0.3 μg/ml, $n=12$). BD plates gave also a different inhibition zone diameter (26±0.5 mm, $n=3$) with respect to DID and OXOID (29±0.5 mm, $n=3$).

Keywords: Agar plates; Mueller–Hinton agar plates; Pharmaceutical analysis; Antibiotics; Ceftazidime

1. Introduction

During microbiological assays, as agar diffusion for antibiotic sensitivity testing, a known amount of drug is placed on the solid culture media and the concentration in agar changes continuously in relation to the time and the distance from inoculation point. High-performance liquid chromatography (HPLC) is often used to quantify the antibiotic concentration in biological samples, but it can also be useful to study the antibiotic kinetics of diffusion in agar in relation to the inhibition zone formation.

The culture media employed in microbiology are mixtures of heterogeneous products derived from animal and vegetal sources (acid digest of casein, dehydrated infusion from beef, cornstarch, etc.). To obtain the solid medium, agar nutrient broth (1.5–2.0%), with its gel-forming properties is added. Agar is a complex polysaccharide prepared from red seaweed harvested all over the world, so agar composition depends on the quality of the seaweed, on the extraction procedure employed [1], and on the kind of impurities included. It is not so surprising, therefore, to find a high variability between composition and physicochemical properties of solid culture media provided by different manufacturers or between lot-to-lot samples produced by the same manufacturer. Variability is more often related to cations concentration into the medium and may hypothetically lead to altered diffusion of antibiotics during the microbiological sensitivity tests. The rate of diffusion of aminoglycosides antibiotics (drugs with a cation molecular structure) in agar is influenced by electrostatic interaction with the acid or sulphate groups of the solid matrix [2]. Moreover, the amount of calcium and magnesium has been shown to stimulate or inhibit the bacterial growth [3,4], while low cation concentrations yielded larger

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inhibition zone sizes and lower minimum inhibitory concentrations (MICs), resulting in a false interpretation of the susceptibility test. Media purchased from different manufacturers displayed a wide range of zinc concentrations: low zinc concentration resulted in low MIC values of imipenem for *P. aeruginosa*, which were normalised by addition of zinc to Mueller–Hinton agar (MHA) [5,6]. On the contrary, it is well known that the MICs of ceftazidime (CAZ) are not influenced by zinc concentration [6].

A complete standardisation of culture media composition has not been achieved until now and a precise control of the drug diffusion process during the susceptibility disc diffusion test, is lacking [7]. The National Committee of Clinical Laboratory Standards (NCCLS) recommends the use of MHA for antimicrobial sensitivity testing, even if this is not a synthetic medium with a completely defined composition. HPLC can be therefore a useful tool to perform a quality assessment of the agar matrix.

In this paper we evaluated using an improved HPLC procedure [8,9] the cation supplemented MHA purchased from three different manufacturers. The microbiological performance in sensitivity tests was also assessed comparing the diffusion of CAZ in agar plates from different sources. A potential relationship between the chromatographic profiles, the CAZ diffusion rate and the variability of the inhibition zone size was also investigated.

2. Experimental

2.1. Chemicals

All the chemicals employed were of analytical grade. Methanol (HPLC grade) was purchased from BDH (Milan, Italy), perchloric acid (PCA) and potassium phosphate bi-basic (K_2HPO_4) were from Fluka (Buchs, Switzerland). The buffers were prepared with bi-distilled water and filtered on 0.45-μm membrane, (Millipore, Bedford, MA, USA) before use.

2.2. Standards

CAZ was from Glaxo (Verona, Italy), theophylline (used as internal standard, I.S.) from Farmitalia-Carlo Erba (Milan, Italy). CAZ solution (1 mg/ml) was prepared in bi-distilled water and stored at −20°C. Weekly, the working solutions were prepared by diluting the stock solutions with water. Theophylline stock aqueous solution (1 mg/ml) was diluted with 2.5% PCA to prepare the working solution (2 μg/ml). The Sensi-disc, susceptibility test discs (CAZ, 30 μg) were all from the same batch (lot No. 1006-612531) purchased from Becton Dickinson (Cockeysville, MD, USA).

2.3. Agar plates

The medium used was, in all cases, MHA. The plates (90 mm diameter) were from Becton Dickinson (BD) (lot No. 0518E74CE), OXOID (lot No. 9248, Milan, Italy) and DID (lot No. 0602706, Milan, Italy).

2.4. Kinetics of diffusion

The standardised disc diffusion test was performed as recommended [7]. The diffusion was studied incubating at 37°C for 2, 6 and 24 h after deposition of the sensitivity discs and, at each incubation time, samples ($n=3$) were harvested at 6 and 12 mm from the centre of the disc. Kinetics were always performed in duplicate and plates discharged after sampling. The data reported for each point are the mean±S.D. of 6 values.

2.5. Agar sampling and CAZ extraction

The samples were harvested as previously described [8,9], employing calibrated glass capillaries (1 mm I.D.) (Coulter Electronics, Luton, UK). Briefly, two agar cylinders were taken at opposite sites of the disc centre, put in an Eppendorf tube, internal standard added (I.S. 200 μl=400 ng) in PCA, heated at 50°C for 15 min, neutralised by adding $NaHCO_3$, centrifuged, filtered and injected into the HPLC column (100 μl).

2.6. Bacterial strain

The studies of CAZ diffusion in the presence of bacterial growth were performed with *P. aeruginosa* (ATCC 27853), adjusting the suspension turbidity to

an optical density of 0.5 McFarland standard (≅1.2·10^8 colony-forming units (CFU)/ml) with sterile 0.85% saline.

2.7. Equipment and chromatographic conditions

A HPLC system (Kontron, Zurich, Switzerland) consisting of two dual-piston pumps (Model 420), an autosampler (Model 460) and a double-beam UV detector (Model 430) was employed. All the modules were controlled by a Model 450 data system for the collection and handling of data. The column was an Ultrasphere ODS (150×4.6 mm, 5 μm) (Beckman Instruments, Palo Alto, CA, USA). Eluent A was 70 m*M* K_2HPO_4, pH 6.5, eluent B was methanol. The column was conditioned with 94% of eluent A and immediately after injection solvent B increased from 6 to 23% over 6 min. Then B was held for 2 min and reported to 6% in 1 min. A new injection was repeated after an additional 6 min. The flow-rate was 1.5 ml/min and the separation was carried out at room temperature monitoring at 255 nm.

2.8. Quantification and statistical analysis

CAZ concentration was calculated as already described [8] on the basis of calibration curves in water and in agar obtained by plotting the peak area ratio (A/A) of CAZ/theophylline vs. the initial CAZ concentration (μg/ml). Standard curves (2–100 μg/ml range) were prepared in BD, DID and OXOID agar media and used to quantify the CAZ kinetics. The differences between CAZ kinetics of diffusion in agar plates from the three manufacturers was evaluated with the Student *t*-test: a p value ≤ 0.05 was considered significant.

3. Results

3.1. HPLC analysis of agar from different sources

The analytical conditions used in this study are improved with respect to our previous studies [8,9]. The LiChrospher C_{18} (Merck) column was replaced with an Ultrasphere C_{18} (Beckman) and a methanol gradient was used instead of an isocratic elution reducing the total analysis time from 20 to 15 min. The CAZ t_r was 4.20±0.08 min while theophylline eluted at 6.73±0.16 min (R.S.D. 2.0%, $n=10$). More than 500 injections were performed without loss of resolution.

Linear regression analysis on aqueous standard curves ($n=5$) gave a slope of 0.0256±0.0003 (mean±S.D.), an intercept of −0.012±0.0002 and $r^2>0.998$. Linearity in agar was assessed in OXOID ($n=5$) (slope 0.0248±0.002, intercept −0.03±0.003, $r^2>0.998$), BD ($n=3$) (slope 0.0269±0.002, intercept −0.014±0.003, $r^2>0.999$) and DID ($n=3$) (slope 0.0265±0.003, intercept −0.027±0.002, $r^2>0.998$) plates. The CAZ recovery from agar was 89.7±9% at the 10 μg/ml level ($n=5$) and the limit of detection was 5 ng injected corresponding to a limit of quantitation in agar of 1.0±0.05 μg/ml ($n=5$). The intra-run precision calculated analysing agar ($n=7$) containing 5 μg/ml of CAZ gave a R.S.D. of 5.0%. For between-day precision the same plates were assayed five days consecutively with a R.S.D.<8.0%.

The HPLC ''fingerprint'' obtained from agar plates by different manufacturers gave quite interesting results (Fig. 1). In OXOID agar extracts many peaks eluted with the void volume while two intense peaks appeared at 3.8±0.3 min and 5.0±0.2 min. A similar profile with minor unretained impurities resulted for the BD agar samples while DID agar showed a remarkable reduction of the peaks at 3.8 and 5.0 min (Fig. 1A). Fig. 1B shows the HPLC profiles of agar samples harvested 24 h after the disc diffusion test for *P. aeruginosa* vs. CAZ. The antibiotic showed a significantly different intensity relative to the manufacturer' agar sample, with a concentration <2 μg/ml in OXOID and DID and 5.1 μg/ml in BD. Moreover, in the presence of bacterial growth, intensity of peaks at 3.8 and 5.0 min was much decreased, while in BD samples a new peak at 8.5±0.3 min resulted (Fig. 1B).

3.2. CAZ kinetics of diffusion

The CAZ kinetics of diffusion in the non-inoculated and inoculated plates from the three manufacturers, are reported in Fig. 2A and B. The CAZ kinetics of diffusion at 6 mm from the disc centre

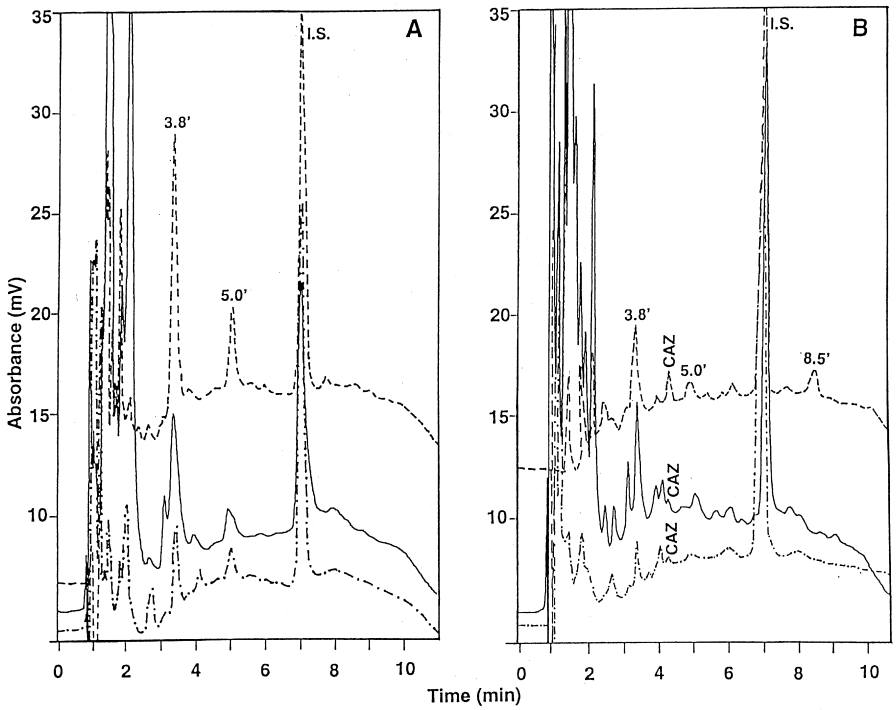

Fig. 1. HPLC profiles of samples extracted from OXOID (———), BD (- - -) and DID (– · –) Mueller–Hinton agar-II plates as described in Section 2. (A) Non-inoculated plates. (B) Disc diffusion test for *P. aeruginosa* (ATCC 27853) vs. CAZ after 24 h at 6 mm from the disc centre.

were very similar in all plates with a maximum concentration peak (60.4±6.7 μg/ml, $n=18$) after 6 h of incubation, and a decrease to 26.8±3.7 μg/ml ($n=18$) at 24 h. A significant difference ($p<0.05$) was found only after 2 h with a CAZ concentration of 8.17±1.0 μg/ml, 12.56±0.5 μg/ml and 17.45±3.2 μg/ml in the OXOID, BD and DID plates, respectively. Kinetics at 12 mm were not statistically different (Fig. 2A).

When the CAZ diffusion was studied in the presence of bacterial growth, the profiles at 6 mm were similar to that of non-inoculated plates, confirming the maximum concentration after 6 h (57.1±4.3 μg/ml, $n=18$). The values measured at 24 h decreased to 1.6±0.3 μg/ml ($n=12$) in OXOID and DID plates (Fig. 2B), while BD samples revealed a CAZ amount (5.1±1.7 μg/ml, $n=6$) significantly higher than in the other two media ($p\leq 0.001$) (Fig. 2B). Moreover, the inhibition zone diameter measured in the BD plates (26±0.5 mm, $n=3$) was different ($p\leq 0.01$) from that found in OXOID and DID (29±0.5 mm, $n=3$), even if all the values were within the NCCLS reference range for *P. aeruginosa* vs. CAZ in the standardised disc diffusion test (22–29 mm). The last observation was that *P. aeruginosa* strain was green dyed only when grown on BD medium.

4. Discussion

With respect to our previously published method [8], the HPLC conditions used in the present work were optimised in order to point out every possible difference among HPLC profiles of agar from various sources. The column now employed, packed with an octadecyl silica (ODS) phase characterised by a maximum coverage bonding and by a final endcapping reaction, together with the gradient elution, allowed to improve the peaks symmetry, the CAZ resolution from interferences and to decrease

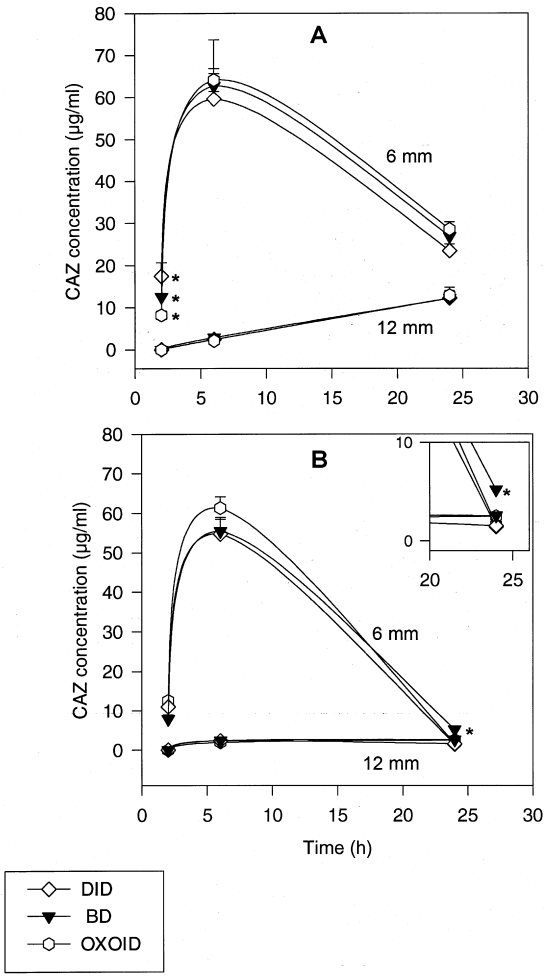

Fig. 2. CAZ kinetics of diffusion in agar plates from different manufacturers. (A) Non-inoculated plates. (B) Inoculated plates. * $p<0.05$.

the total analysis time with an amplified daily workload.

MHA is recommended for the growth of a wide class of microorganisms [1,11] due to its limited shift of pH during the microbial growth and low concentration of sulphonamide and tetracycline inhibitors. Nevertheless, the variability in the performance of MHAs from different manufacturers has been already shown to be statistically significant [10] and today manufacturers are required to evaluate MHA as reported in the ''Approved Standard'' document of NCCLS [11]. Factors contributing to this variability have not been investigated yet and, for this purpose, our chromatographic technique may be considered a very useful tool in the development of a quality control program for agar plates. In our study, the HPLC analysis evidenced a different ''fingerprint'' in relation to the kind of agar and/or nutrients employed, even before the disc susceptibility test execution. These qualitative differences were amplified by the presence of the microorganism confluent growth, when the inhibition zone is well defined. At 24 h, all the chromatographic profiles showed a dramatic reduction of many peaks which could be representative of nutrients metabolised by the bacteria during their growth.

In non-inoculated plates, the remarkable different concentration found after 2 h, probably was partially due to the medium viscosity (low in OXOID and higher in DID) which caused a different migration rate of the drug in the first hour of incubation. As diffusion progressed, the concentration of the antimicrobial agent, at 6 mm, increased, reaching a maximum level after 6 h. At this time, and up to 24 h, the variability of the diffusion rate due to the matrix composition was less remarkable.

In the presence of bacterial growth, BD plates displayed different behaviour with a CAZ concentration at 24 h higher than in the other media and justified by the 3 mm smaller diameter of the inhibition zone. This was also in agreement with the higher MIC value (1.5 µg/ml instead of 1.0 µg/ml as for OXOID and DID) previously found by us on Etest® (unpublished observation). This finding evidenced that quality control of antibiotic kinetics of diffusion in the presence of the reference strains (in our test *P. aeruginosa* ATCC 27853), allowed a more rigorous evaluation of the medium performance, being the inhibition zone the resultant of the antibiotic kinetics and the bacterial growth.

Cooper et al. [6] described the significant effect of the concentration of calcium, magnesium and zinc, in MHA, on the susceptibility of *P. aeruginosa* to imipenem, reporting higher MIC values for BD, with a high zinc concentration, with respect to DID MHA plates. On the contrary, the activity of cephalosporins and in particular of CAZ, against *P. aeruginosa*, was not affected by the zinc concentration. Our research on *P. aeruginosa*, however, was able to evidence a reduced size of the inhibition zone on BD plates and, in addition, to confirm this observation [3–6,10] with

a quantitative analysis. In this study all the agar plates led to a correct classification although the *P. aeruginosa* grown on BD agar could seem ''more resistant''. In particular, the observed variability in diffusion and inhibition zone, might induce a false clinical conclusion for the unknown ''intermediate'' bacterial strains. In fact, if a *P. aeruginosa* clinical strain shows an inhibition zone diameter against CAZ in the 14–18 mm range or MIC values between 32 and 8 μg/ml (intermediate sensitivity level), a difference of 3 mm in the diameter or of two dilution in broth MIC, may change the classification from intermediate to susceptible or to resistant. HPLC evaluation of antibiotic concentration in agar may be therefore important for a more precise quality control of the plates used in agar diffusion antimicrobial susceptibility test, or in the recent instrumentation able to supply the MIC values by direct reading of the inhibition zone diameters. HPLC quantification of antibiotic in agar could be useful to understand the basis of microbiological tests variability, decreasing the risk of a wrong classification.

In order to improve reproducibility, it would seem advisable, as emphasised in the NCCLS document [11], to have a protocol which would permit to compare each batch of culture medium routinely produced with a reference one.

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