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Journal of Chromatography B, 753 (2001) 151–156

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Quantification of gentamicin in Mueller–Hinton agar by high-performance liquid chromatography

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Abstract

The aim of this study was to optimise a method for gentamicin determination in an agar matrix and to investigate if and how agar composition can affect the gentamicin diffusion kinetics during the agar diffusion tests for antibiotics sensitivity. Gentamicin was separated by RP-HPLC and detected at 365 nm after pre-column derivatization with 1-fluoro-2,4-dinitrobenzene. Recovery (≥79%), linearity (r^2≥0.997) and sensitivity (1 μg/ml) were assessed using four different agar matrices. The kinetics of gentamicin diffusion tested on BioMerieux and DID manufacturers' products showed in uninoculated agar plates significant differences that were even more pronounced in the presence of *Pseudomonas aeruginosa* metabolism.

Keywords: Mueller–Hinton agar plates; Gentamicin

1. Introduction

Studies of antibiotic diffusion in agar and of the effects of the different composition of this medium (pH, cation concentration, supplements) have been undertaken [1–6]. The wide variability between culture media from different manufacturers mainly derives from the different mixture of animal and vegetable components added to agar polysaccharide [7,8].

The quality control (QC) of agar matrix is mainly related to its performance in microbiological tests, like the inhibition zone diameter obtained in standardised conditions from a couple of antibiotic/microorganism combinations in the disc diffusion sensitivity test (Kirby–Bauer test) [9–12]. However, this parameter represents only an indirect estimation deriving from the results of the antibiotic diffusion and bacterial growth kinetics. To have more precise knowledge on the quality of agar from different manufacturers, drug diffusion should be evaluated in the absence of bacterial growth.

We previously focused on the importance of this standardisation, studying the performance of ceftazidime (a cephalosporin) in agar using high-performance liquid chromatography (HPLC) [13–15].

This research, as part of a wider project aimed at comparing the behaviour of different antibiotic families in agar, concerns gentamicin. It is well known that the rate of diffusion of aminoglycosides is strongly influenced by variations in agar composition. In particular, the acid sulfate groups of the solid matrix [16] and magnesium and calcium content [7,8,10,17] interacting with these antibiotics,

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0378-4347/01/$ – see front matter
PII: S0378-4347(00)00460-6

would cause a slackening in their agar diffusion resulting in a reduction of diameter width.

The HPLC method proposed here for the direct quantification of gentamicin in agar was validated employing agar from four different manufacturers and was used to compare the gentamicin kinetics of diffusion in the presence and in the absence of *Pseudomonas aeruginosa* ATCC 27853 strain. Two different manufacturers were also compared in terms of inhibition zone diameters.

2. Experimental

2.1. Chemicals

All the chemicals employed were of analytical grade. Acetonitrile (HPLC grade) was purchased from BDH (Milan, Italy), tris(hydroxymethyl)aminomethane (Tris) was from Sigma–Aldrich (Milan, Italy) and 1-fluoro-2,4-dinitrobenzene (FDNB) was from Merck (Bracco, Milan, Italy). Gentamicin sulfate (potency: 684 μg/mg) was a gift of Schering Plough (Milan, Italy).

2.2. Drug standards

Gentamicin solution (2 mg/ml) was prepared in bidistilled water and stored at −20°C.

2.3. HPLC analysis

A Kontron (Zurig, Switzerland) HPLC apparatus and an Ultrasphere ODS column (250×4.6 mm) (Beckman Instruments, Palo Alto, CA, USA) were employed. Eluent A was Tris (8.25 m*M*) prepared with bidistilled water and adjusted to pH 7.0 with 1 *M* hydrochloric acid. Eluent B was acetonitrile. The mobile phase was filtered and degassed on a 0.45-μm membrane (Millipore, Bedford, MA, USA), before use. The column was conditioned with 75% of eluent B and immediately after the injection (150 μl) eluent B was decreased to 73% in 16 min. Then B was returned to 75% in 1 min and a new injection was repeated after 1 min. The flow-rate was 1.3 ml/min and the separation was monitored with UV detection at 365 nm.

2.4. Gentamicin extraction and derivatization

A previously published analytical method for gentamicin in serum [18] was optimised for determination in agar matrix. Four agar cylinders (3 μl each), harvested with calibrated glass capillaries (1 mm I.D.) (Coulter Electronics, Luton, UK), or, in the case of aqueous standards, 12 μl of working solution, were placed in an Eppendorf tube. Acetonitrile (100 μl), Tris (165 m*M* in bidistilled water: 50 μl) and FDNB (269 m*M* in acetonitrile: 20 μl) were added. After heating at 80°C for 15 min, 150 μl was injected into the HPLC column.

2.5. Linearity and quantification

The linearity of the method was tested in aqueous working solutions (0.5, 1, 5, 10, 50, 100 μg/ml) obtained by dilution from the 2 mg/ml standard and analysed by HPLC. Linearity in agar was evaluated for all the manufacturers' materials by preparing agar plates (in triplicate) with known and homogeneous concentrations of gentamicin (1, 10, 50, 100 μg/ml) as already described [13]. Agar cylinders were harvested, treated as described for unknown samples and the slope of the calibration curves calculated by linear regression analysis, plotting gentamicin area vs. agar concentration.

2.6. Agar plates

The Mueller–Hinton Agar (MHA) II plates (90 mm diameter) were from: Becton Dickinson (BD) (lot No. E83AA, Cockeysville, MD, USA); Oxoid (lot No. B3374, Basingstoke, UK); BioMerieux (lot No. 719867401, Marcy-l'Etoile, France) and from DID (lot No. 0602706A, Milan, Italy).

2.7. Kirby–Bauer test

Kinetics of diffusion in presence of bacterial growth were performed with *P. aeruginosa* ATCC 27853. The suspension turbidity was adjusted to an optical density of 0.5 McFarland standard (~$1.2 \cdot 10^8$ CFU/ml) with sterile saline solution and the disc

susceptibility test (Kirby–Bauer) was performed as recommended [10].

2.8. Kinetics of diffusion

The antibiotic diffusion was studied in agar from DID and BioMerieux manufacturers as a function of time from the disc deposition and the distance from the disc edge. All the distances, for each incubation time, were sampled from the same plate. A gentamicin sensi-disc (10 μg) was put on the centre of inoculated and uninoculated plates and agar was sampled after 2, 4, 6, 15 and 24 h of incubation at 37°C from opposite sites of the disc centre at 3, 6 and 9 mm [15]. Kinetics were always performed in triplicate. The sensi-discs were all from the same batch (lot No. B1006-708545) and were purchased from Becton Dickinson.

2.9. Statistical analysis

The differences in gentamicin agar diffusion for the four manufacturers were evaluated performing the Student *t*-test: a P value ≤ 0.05 was considered significant.

3. Results

3.1. Validation of the HPLC method

Under the described analytical conditions the antibiotic showed five peaks belonging to gentamicin sulfate derivatives (C1, C1a, C2, C2a, C2b) [19–23] (Fig. 1A). To evaluate the gentamicin kinetics of diffusion we took into account only the first peak eluting at 8.40±0.042 min (RSD=0.5%, $n=10$) although we found a good correlation and linearity

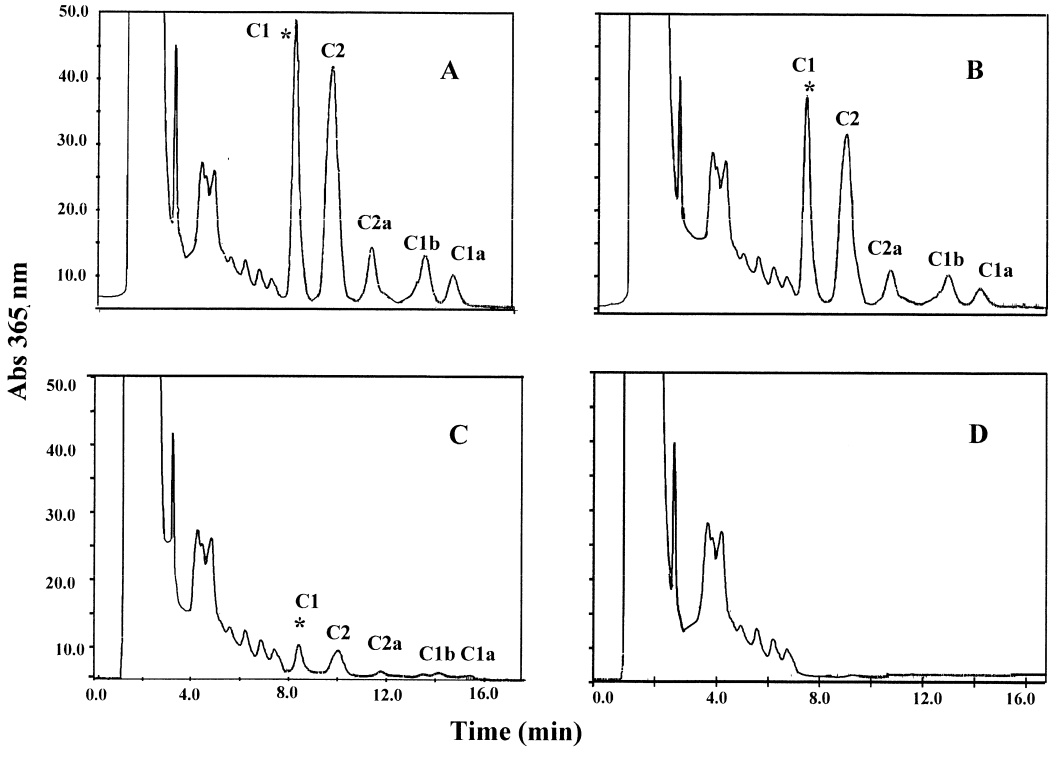

Fig. 1. HPLC profiles of a gentamicin standard curve in DID agar matrix. (A–C) Agar enriched with gentamicin standard (100, 50, 10 ng/μl, 5.0, 2.5, 0.5 μg injected, respectively). (D) Agar without gentamicin addition. The samples were treated and analysed by HPLC as described in the Experimental section except flow-rate was 1.2 ml/min. *Gentamicin peak at t_R 8.40 min taken into account to evaluate antibiotic kinetics of diffusion. C1, C2, C2a, C2b and C1a, gentamicin derivatives.

between the peak area response and the standard concentration for all the peaks detected. The area of each peak, expressed as the percentage of the sum of the peak areas, corresponded to the drug composition (expressed in percent of each gentamicin derivative) furnished by the Schering-Plough QC analysis. From these data and on the basis of the chemical structures of the components, we identified C1 as the peak at t_R 8.40 min (34%), C2 (t_R~10 min, 43%), C2a (t_R~12 min, 9.8%), C2b (t_R~14 min, 8.3%) and C1a (t_R~16 min, 4.9%) (Fig. 1A).

Linear regression analysis on aqueous standard ($n=3$) gave a slope of 0.184±0.001 (mean±SD), an intercept of −0.230±0.11 and $r^2=0.999$. Linearity in agar assessed in BD (slope 0.157±0.009, intercept 0.55±0.017, $r^2=0.997$, $n=3$), Oxoid (slope 0.147±0.02, intercept 0.11±0.08, $r^2=0.999$, $n=3$), BioMerieux (slope 0.159±0.0047, intercept 0.59±0.09, $r^2=0.999$, $n=3$) and DID (slope 0.148±0.01, intercept 0.25±0.05, $r^2=0.999$, $n=3$) matrices did not show significant differences. The recoveries from agar, evaluated in the 1–100 μg/ml ($n=10$) range by comparison with aqueous standards, were 85±5.5% for BD, 79±4.0% for Oxoid, 86±3.4% for BioMerieux and 80±4.25% for DID.

The intra-run precision calculated analysing the different agars at known concentration (10 μg/ml, $n=6$) gave an RSD=6.52%, while between-day precision, evaluated on the same plates for 3 consecutive days, gave an RSD=7.69% ($n=9$).

The sensitivity of the method was determined assuming a signal/noise ratio of 3 and was estimated to be 9.89 ng injected onto the HPLC system. This level corresponds to 1 μg/ml in agar medium.

3.2. Kinetics of diffusion in agar plates

The kinetics in the absence and in the presence of bacterial growth were performed on DID and BioMerieux plates, focusing on concentrations at 9 mm from the disc centre. Fig. 2 reports the results obtained. After 15 h of incubation in the presence of *P. aeruginosa*, gentamicin levels were significantly lower than those found in the uninoculated plates in both the matrices considered. In addition, HPLC analysis also revealed significant differences between the two manufacturers (4.65±0.48 vs. 3.51±0.65 μg/ml, DID vs. BioMerieux) that were amplified after 24 h (3.37±0.42 vs. 1.20±0.55 μg/ml, DID vs. BioMerieux). The inhibition zone diameter in the presence of *P. aeruginosa* ATCC 27853 vs. gentamicin was 17.06±0.11 mm (RSD=0.64%, $n=3$) on BioMerieux plates, and 25±0.10 mm (RSD=0.40%, $n=3$) on DID plates ($P\leq0.05$). Despite the observed differences, the residual concentration (1.01±0.05 μg/ml) found by HPLC at the inhibition zone edge in DID plates (12.5 mm from the disc centre) was similar to that found in BioMerieux at 9 mm from disc centre.

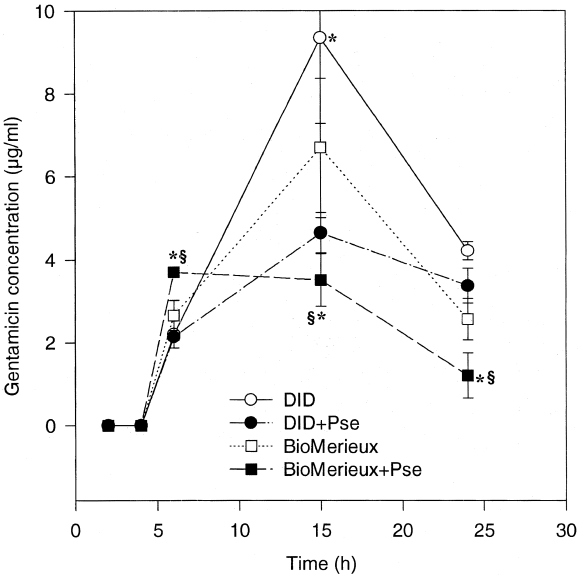

Fig. 2. Gentamicin kinetics of diffusion on DID and BioMerieux plates in the presence and in the absence of bacterial growth (*P. aeruginosa* ATCC 27853). The samples were harvested at 9 mm from the disc centre. *$P<0.05$ inoculated vs. uninoculated plates of the same manufacturer. §$P<0.05$ BioMerieux inoculated plates vs. DID inoculated plates.

Fig. 3 shows the chromatograms obtained analysing samples from uninoculated and inoculated BioMerieux and DID plates, after 15 h of incubation at 9 mm from the disc centre. The comparison of HPLC profiles shows a lower amount of the antibiotic concentration traceable in agar in the presence of microorganisms (4.65±0.48 μg/ml vs. 9.35±2.08 μg/ml for DID plates and 3.51±0.64 μg/ml vs. 6.69±1.68 μg/ml for BioMerieux plates, at 15 h and 9 mm). In addition, the reduction or the disappearance of many other peaks, representative of culture

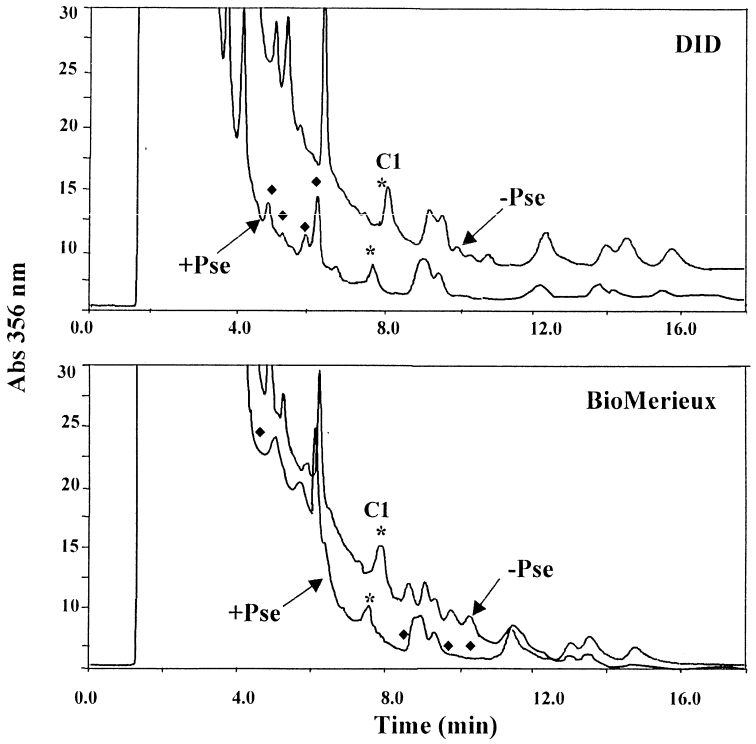

Fig. 3. HPLC profiles of agar samples from BioMerieux and DID plates in presence (+Pse) and in lack (−Pse) of bacterial growth. Plates were incubated for 15 h and sampling was done at 9 mm from the disc centre. *C1: Gentamicin C1 peak. ♦ Peaks corresponding to agar matrix components reduced by bacterial metabolism.

media nutrients metabolised during bacterial growth is impressive.

4. Discussion

Gentamicin determination by HPLC has been described both with fluorimetric [19–21] and UV detection [18,22]. For our purpose, UV detection coupled to pre-column derivatization with FDNB was chosen. Under the described conditions we were able to separate all of the five gentamicin derivatives in a short analysis time. Moreover, the use of FDNB allowed us to automate the analysis by preparing large batches of samples which were analysed overnight without loss in peak area response as we have previously noted by treating the samples with *o*-phthalaldehyde (OPA) (data not shown).

The HPLC analysis of agar samples from different manufacturers indicated the matrix influence on the antibiotic diffusion even in the absence of bacterial growth. In the presence of bacterial growth, gentamicin concentration at 15 and 24 h was lower ($P<0.05$) than the one found in the uninoculated plates due to the *P. aeruginosa* metabolism.

According to Barry and Effinger [24] if the bacteria grow more rapidly and better because the agar has a higher nutrition capacity, inhibition zones tend to be smaller. With this vision we can conclude that the combination of antibiotic diffusion kinetics and bacterial growth in plates we examined could have caused the observed differences in the Kirby–Bauer test.

Pollock et al. [8], moreover, documented how behaviour of standard microorganisms can be transposed to bacteria isolated from patients. According to this view we hypothesise that microorganisms isolated from patients could produce on DID plates inhibition zones larger than on BioMerieux plates, leading to a wrong classification. The performance of

DID agar demonstrates how important it is to have standardised procedures and to employ materials in conformity with QC to obtain reliable results in diagnosis. Moreover, the inhibition zone diameter measurements in the presence of reference strains is a standardised QC procedure able to give a general view on the performance of the system, but unable to demonstrate alterations of the single components.

The HPLC method, validated on four different agar matrices, may be useful to agar producers and control agencies to improve the QC of sensitivity test media. The application of kinetics of diffusion methods on commercial MHA matrix compared with that of the reference MHA National Committee for Clinical Laboratory Standards, may help to better understand the problems related to the composition of the sensitivity test media. Our data stress the necessity to set up new methods employing techniques different from the microbiological ones, to define a ''reference'' agar matrix able to improve the precision and to furnish standardised results in agar diffusion tests.

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