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Article

Biological and physicochemical stability of ceftazidime and aminophylline on glucose parenteral solution

Carolina Alves dos Santos*, Laura Oliveira-Nascimento, Marcos Camargo Knirsch, Marco Antônio Stephano, Adalberto Pessoa Júnior, Thereza Christina Vessoni Penna

School of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP

Ceftazidime is a broad spectrum antibiotic administered mainly by the parenteral route, and it is especially effective against *Pseudomonas aeruginosa*. The period of time in which serum levels exceed the Minimum Inhibitory Concentration (MIC) is an important pharmacodynamic parameter for its efficacy. One of the forms to extend this period is to administer the antibiotic by continuous infusion, after prior dilution in a Parenteral Solution (PS). The present work assessed the stability of ceftazidime in 5% glucose PS for 24 hours, combined or not with aminophylline, through High Performance Liquid Chromatography (HPLC). The physicochemical evaluation was accompanied by in vitro antimicrobial activity compared MIC test in the 24-hour period. *Escherichia coli* and *Pseudomonas aeruginosa* were the microorganisms chosen for the MIC comparison. The HPLC analysis confirmed ceftazidime and aminophylline individual stability on PS, while the MIC values were slightly higher than the mean described in the literature. When both drugs were associated in the same PS, the ceftazidime concentration by HPLC decreased 25% after 24 hours. Not only did the MIC values show high loss of antibiotic activity within the same period, but also altered MIC values immediately after the preparation, which was not detected by HPLC. Our results indicate that this drug combination is not compatible, even if used right away, and that PS might not be the best vehicle for ceftazidime, emphasizing the importance of the MIC evaluation for drug interactions.

Uniterms: Drug/interactions. Injectables. Parenteral solutions. Ceftazidime/stability. Aminophylline/stability. High Performance Liquid Chromatography/quantitative analysis. Minimum inhibitory concentration (MIC).

Ceftazidima é um antimicrobiano administrado por via parenteral, que apresenta amplo espectro de ação, principalmente contra *Pseudomonas aeruginosa.* O tempo em que a concentração sérica de ceftazidima permanece acima da concentração mínima inibitória (MIC) é um importante parâmetro farmacodinâmico para a determinação da eficácia antimicrobiana e pode ser potencializado através da utilização de infusão contínua em soluções parenterais (PS). Este artigo visa a avaliar a estabilidade da ceftazidima em solução de glicose 5%, na presença e na ausência do fármaco aminofilina, através de cromatografia líquida de alta eficiência HPLC e MIC durante o período de 24 horas. Os microorganismos selecionados para a determinação do MIC foram *Escherichia coli* e *Pseudomonas aeruginosa.* Os ensaios em cromatógrafo líquido confirmaram a estabilidade dos fármacos ceftazidima e aminofilina quando são individualmente associados em PS, enquanto os valores de MIC ficaram maiores que os valores encontrados na literatura. Quando ambos os fármacos foram associados na mesma solução parenteral a concentração de ceftazidima obtida por HPLC diminuiu 25% depois de 24 horas. Os valores de MIC mostraram maior decaimento da atividade antimicrobiana neste mesmo período e também valores de MIC alterados nas soluções preparadas no tempo zero, decaimento este que não foi detectado em HPLC. Os resultados indicaram incompatibilidade na associação dos fármacos em PS, enfatizando a importância dos resultados de MIC para interações de fármacos.

Unitermos: Fármacos/interação. Injetáveis. Soluções parenterais. Ceftazidima/estabilidade. Aminofilina/estabilidade. Cromatografia Líquida de Alto Desempenho/análise quantitativa. Concentração Inibitória Mínima (MIC).

*Correspondence: C. A. Santos. Faculdade de Ciências Farmacêuticas, Universidade de São Paulo. Av. Professor Lineu Prestes, n.580, Bloco 16, 05508-000 - São Paulo - SP, Brazil. E-mail: carolinasantos@usp.br

INTRODUCTION

The most common route for drug administration in hospitals is the parenteral one. Parenteral solutions are used as vehicles for drug administration, a procedure that might include more than one drug diluted in one solution. This combination favors drug-drug interactions and occasionally results in less effective drugs or undesired side effects (Trissel, 2010). Another procedure that gives room for drug instability is the extension of the infusion period with the same parenteral bag. When an antibiotic is the drug to be administered, it may lead to damage beyond inefficacy for an individual patient. Suboptimal antimicrobial doses can contribute to microbial adaptation and survival, which allows contamination of the hospital environment with antibiotic-resistant bacteria (Jacobs, 2003). Therefore, it is essential to evaluate the stability of antibiotics under the same conditions used in the hospital, in order to guarantee the therapeutic effect and prevent microbial resistance.

Ceftazidime (Figure 1A) is a β-lactam antibiotic widely used in hospital care through the parenteral route to treat gram-negative infections (Dumartin *et al.*, 2010). The antibiotic is highly efficient against *Pseudomonas aeruginosa*, which is the leading cause of hospital-acquired infections (Amyes *et al.*, 1994). Although specific, its plasma half-life is short, with an average of 2 hours (El-Shaboury *et al.*, 2007), considering patients with preserved renal function (Dalen *et al.*, 1986). Ceftazidime belongs to the third-generation cephalosporin class, which has maximum *in vivo* activity when the concentration is four times the Minimum Inhibitory Concentration (MIC) (Mouton, Vinks, 1996). Due to short plasma half-lives, the bactericidal properties from β-lactam antibiotics are dependent on the time that its concentration remains above the MIC (Fantin *et al.*, 1994). The maximum ceftazidime activity can be sustained over time if: the standard dose is increased; it is given more frequently; or it is administered by continuous infusion (van Zanten, 2009). Continuous infusion of ceftazidime was proved to be effective against gram-negative infections, including resistant *P. aeruginosa* and other multidrug resistant strains (Nicolau *et al.,* 2001; Lorente *et al.,* 2007; Moriyama *et al.*, 2009; Moriyama *et al.*, 2010). The choice of continuous infusion can also decrease the antibiotic daily dose (Nicolau *et al.,* 1996) and result in better cost-effectiveness (McNabb *et al.,* 2001). Nevertheless, it is still controversial which dose regimen is the best, due to limited human studies on the subject (Mouton, Vinks, 1996; Roberts *et al.*, 2009).

The antimicrobial properties of ceftazidime can be associated with the bronchodilator properties of aminophylline (Figure 1B), which may improve the treatment of patients with respiratory disorders (Pleasants *et al.*, 1992). Aminophylline is more effective when administered to hospitalized patients through continuous infusion (Budavari *et al.*, 1996; Hilliard *et al.*, 2000), probably due to significant differences in the plasma half-life of theophylline over time (Kubo *et al.,* 1986). The combination of both drugs was reported as stable for up to two hours in parenteral solutions (Pleasants *et al.*, 1992). Since most hospitals do not submit the drug mixture to a rigorous quality control, the association should be carefully studied according to its physicochemical and biological properties. Based on the presented information, this work aimed to evaluate the stability of the ceftazidime-aminophylline association in glucose parenteral solution. The physicochemical stability evaluation was carried for 24 hours by HPLC (High Performance Liquid Chromatography) and the antimicrobial activity was analyzed during the same period by the MIC (Minimum Inhibitory Concentration) test.

MATERIAL AND METHODS

Material and reagents

Aminophylline (99.8 % Medley, Brazil) and ceftazidime (99.9 % anhydrous base) were used as the secondary

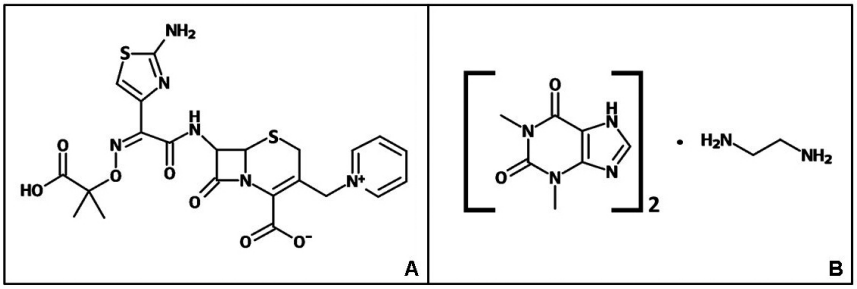

FIGURE 1 - Molecular structure of ceftazidime (A) and aminophylline (B). A – Ceftazidime: $C_{22}H_{22}N_6O_7S_2$. B – Aminophylline: two molecules of theophylline, $C_7H_8N_4O_2$, for each molecule of ethylenediamine, $C_2H_4(NH_2)_2$.

standards for HPLC analysis. All glassware used in the experiments were previously immersed in a 1 M NaOH solution for 24 h, drained and transferred to a 1 M nitric acid solution for 6 h. After extensive rinsing and 24 h immersion in purified water (Milli-Q), the equipment was autoclaved for 30 min at 121 °C. All plastic materials were sterile and disposable. All reagents used were of analytical grade.

Drug solutions

Ceftazidime (Glaxo Smithkline, Brazil) was diluted (0.1 mg/mL) in a parenteral solution with 5% glucose (Aster, Brazil) for the stock solution. The same was made for aminophylline (Hypofarma, Brazil) to a final concentration of 24 mg/mL. Stock solutions were made immediately prior to use at room temperature (25 ºC). The stock solutions were then diluted to 320µg/mL for ceftazidime and 160 µg/mL for aminophylline in parenteral solution, individually or associated with each other (herein called "drug mixture"). The chosen dilutions were based on the plasma concentrations reported in the literature (Trevor, Katzung, Masters, 2001).

Bacterial strains and growth conditions

Escherichia coli ATCC 25922 and *Pseudomonas aeruginosa* ATCC 9721 were used as the biological sensors for antimicrobial activity. *E. coli* was inoculated in Luria Bertani (LB) (DIFCO) broth medium and cultivated at 37 ºC/100 rpm/24 hours. *P. aeruginosa* was inoculated in Trypticase Soy Broth (TSB) broth and also cultivated at 37 ºC/100 rpm/24 hours.

pH Determination

The pH of all solutions was measured at 25 °C with a pHmeter (Accumet AR20, Fisher Scientific, USA) before analysis by HPLC and MIC test.

HPLC

Pharmacopeic methods (USP, 2009) of ceftazidime and aminophylline were combined in a new method described in this item. Reversed-phase HPLC was performed on a Shimadzu LC 10 (software LC solution; Shimadzu, Japan), using a 250 x 4.6 mm (internal diameter) column pre-packed with C18 and 12.7 nm of pore size (Shim-pack VP–ODS, Shimadzu, Japan). The parameters applied were 0.5 mL/min flow at 25 °C and a sample injection volume of 20 µL, while the mobile phase consisted of 35% acetonitrile, 2% phosphoric acid 1% (pH 7.0) and water (WFI - Water For Injection). Eluted samples were detected at the optimum wavelength (Budavari *et al.*, 1996) for ceftazidime (255 nm) and aminophylline (275 nm). This method was developed according to the validation guideline from the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (ICH, 2005). The required assays described in the guideline are specificity, linearity and range concentration, accuracy, precision and reproducibility. In order to access specificity in the presence of degradation products, the samples were exposed to 10% H_2O_2 (2:1, v/v) for 1h, followed by HPLC analysis and comparison with the untreated samples.

MIC determination

The antimicrobial activity of ceftazidime samples was evaluated by MIC test, according to the guideline from the Clinical and Laboratory Standards Institute (CSLI) (CSLI, 2009). Briefly after the cultivation, both microorganisms were adjusted to 10^6 Colony Forming Units (CFU)/mL and distributed in test tubes with two-fold serial dilutions of samples, except for the negative controls. Tubes were then incubated for 24 h/37 °C. Bacterial counts were made in agar plates cultured with the content of the test tubes for 24 hour incubation at 37 °C.

Data analysis

Chromatograms were obtained using the LC Solution Software (Shimadzu). To confirm the linearity of the HPLC method, a regression line was calculated by the least-squares method. To check the statistical significance of the differences (p) between the mean values compared throughout the article, the two-tailed t-test method was applied using the Excel® software, where p values below 0.05 represent statistical significance of the differences between two compared means.

RESULTS

pH Determination

The pH values of all solutions were within the ranges described in the literature. The observed values did not change after 24-hour storage at room temperature. The ceftazidime average pH was 6.6, with standard deviation of 0.3 (pH=6.6 ± 0.3). The aminophylline average pH=9.5 ± 0.3 and the ceftazidime + aminophylline average pH=8.30 ± 0.4.

HPLC method development and performance

The HPLC method presented no statistical differences between the retention times for samples and for the respective standards (ceftazidime p=0.13 and aminophylline p=0.06). In addition, peroxide degradation products did not interfere with non-degraded drug peaks (Figure 2). Due to both facts, the developed methodology can be considered specific. The method was linear for both substances, with R^2 of 0.99 in the 70-130% range. Accuracy and precision were also achieved, with standard deviation less than 0.1 % of the sample value. There were no inter-laboratorial assays; the reason why reproducibility was not verified.

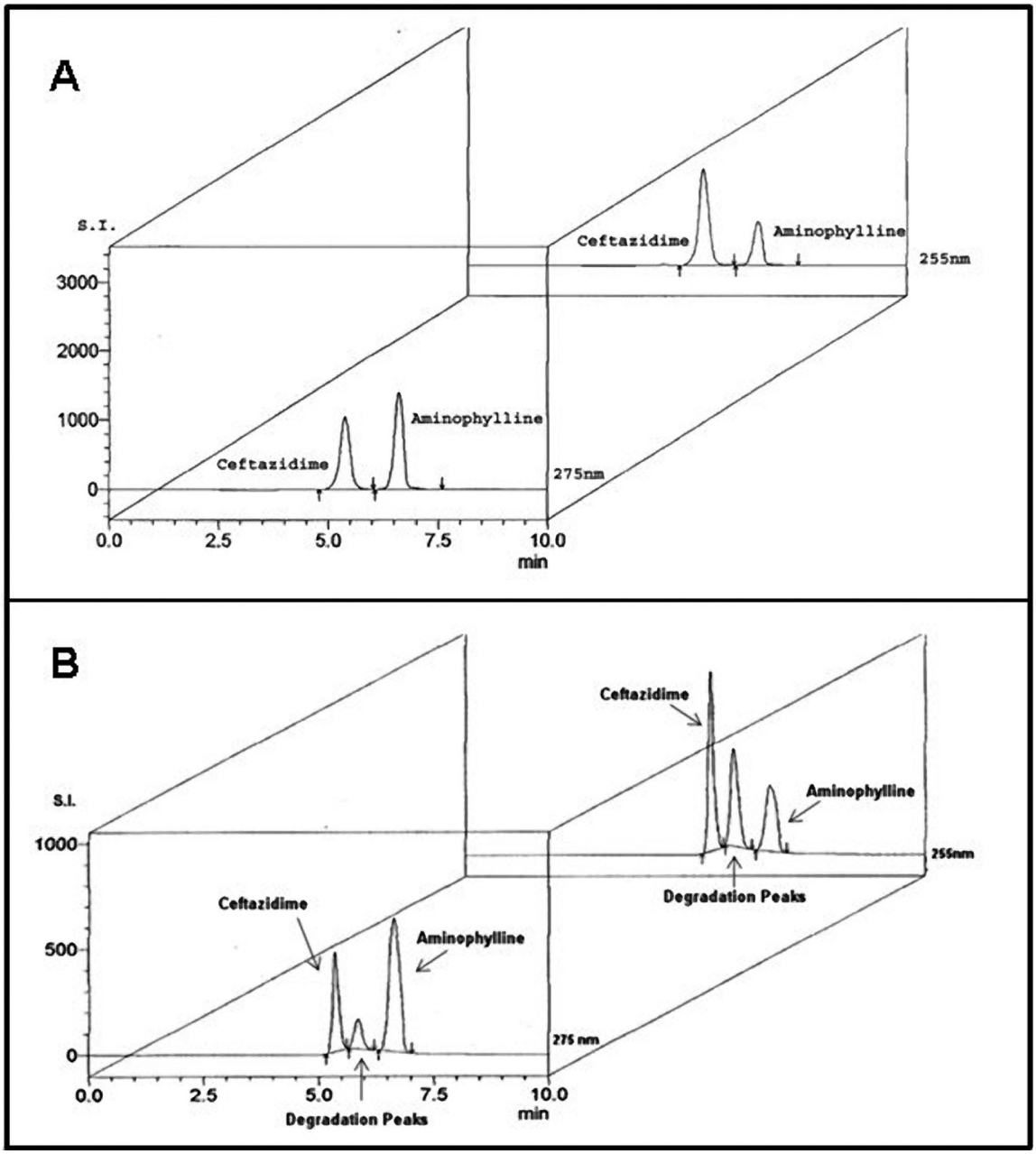

FIGURE 2 - Specificity of the HPLC method for ceftazidime and aminophylline. A) Chromatograms of ceftazidime (320 µg/mL) and aminophylline (160 µg/mL) untreated samples. B) Chromatograms of the same samples treated with hydrogen peroxide. Samples were read at 275 nm, the ideal wavelength for aminophylline detection (front chromatogram of A and B), and at 255 nm, the ideal wavelength for ceftazidime detection (back chromatogram of A and B). S.I.=Signal intensity, min=minutes (retention time).

Stability of ceftazidime and aminophylline solutions by HPLC

Triplicate samples of ceftazidime, aminophylline and the drug mixture were diluted in 5% glucose parenteral solutions, followed by HPLC analysis at 0, 2, 6 and 24 h after preparation. Drugs were considered stable when concentrations were ≥95 % of the values obtained immediately after drug dilution, without mixing (Farina *et al.,* 1999). Aminophylline, was stable at all time periods evaluated (Table I), as it was previously published, regardless of ceftazidime addition.

TABLE I - Stability of aminophylline diluted in 5% glucose parenteral solution*

Time (h)	Aminophylline (ug/mL)* - drug mixture	Aminophylline (ug/mL)* - individual solution
0	167.94	165.37
2	168.50	164.47
6	170.90	167.40
24	169.63	164.70

*The reported mean values were collected from three independent experiments, using triplicate samples in each of them. The highest standard deviation was less than 0.02% of the mean concentration value.

Ceftazidime was also stable when diluted alone. However, its association with aminophylline resulted in a 12.5% loss of the antibiotic after the 6 h period (Table II). The decrease in concentration doubled at 24 h (25%), confirming the instability reported for this association (El-Shaboury *et al.*, 2007).

Antimicrobial activity of ceftazidime solutions by the MIC test

Ceftazidime, aminophylline and the drug mixture were diluted in 5% glucose parenteral solutions, followed by MIC determination at 0 and 24 h after preparation

TABLE II – Stability of ceftazidime diluted in 5% glucose parenteral solution*

Time (h)	Ceftazidime (ug/mL)* - drug mixture	Ceftazidime (ug/mL)* - individual solution
0	320.44	325.80
2	301.54	322.86
6	280.81	322.24
24	243.86	311.37

* The reported mean values were collected from three independent experiments, using triplicate samples in each of them. The highest standard deviation was less than 0.02% of the mean concentration value.

(Table III). The tested bacteria were *E. coli*, a standard sensitive strain, and *P. aeruginosa*, the main target pathogen for this antibiotic. The observed MIC for ceftazidime alone at time zero is within the range found in the literature, but slightly higher than the available means (Neu, Labthavikul, 1982). This MIC value was herein considered as the reference value for comparison purposes (1.0 µg/mL of ceftazidime for *E. coli* and 1.9 µg/mL for *P. aeruginosa*). The MIC value for ceftazidime alone increased four times for *E. coli* and two times for *P. aeruginosa* after 24 h, showing a decrease of the biological activity. The drug mixture revealed negative interactions immediately after the preparation, with a MIC value 15 times higher than the reference value for *E. coli*. This effect was less prominent in the test with *P. aeruginosa*, in which the MIC was 4 times higher than the reference. The analysis after 24 h showed MIC values 120 times higher than the reference for *E. coli* and 15 times higher for *P. aeruginosa*. The aminophylline solution was not bactericidal by itself, as expected.

DISCUSSION

The physicochemical stability of ceftazidime has been analyzed by HPLC in a variety of solutions. This antibiotic was previously reported as stable in 5% glucose

TABLE III – Antimicrobial activity of ceftazidime diluted in 5% glucose parenteral solution

Strain	MIC ceftazidime (µg/mL)* - (individual solution)		MIC ceftazidime (µg/mL) - Drug mixture*	
	0 h	**24 h**	**0 h**	**24 h**
Escherichia coli	1.0	3.7	15.0	120.0
Pseudomonas aeruginosa	1.9	3.7	7.5	30.0

* The reported mean values were collected from three independent experiments, using triplicate samples in each of them. The highest standard deviation was less than 0.01% of the mean concentration value.

solution after 24 hours, although a 5% loss was detected (Moriyama, 2009). The same profile was confirmed in our results.

The spread of HPLC analysis contrasts with the rarity of antimicrobial activity tests applied to ceftazidime stability studies. Our efforts were then directed to determine its antimicrobial activity in the same glucose parenteral solution. MIC values of ceftazidime that inhibit *P. aeruginosa* growth doubled after 24 hours. Since MIC determination is based on a serial 2 fold dilution, the 5% antibiotic loss detected by HPLC could double the MIC value.

A prominent loss in antimicrobial activity of ceftazidime was observed against *E. coli*, where the MIC increased four times after 24 hours. This increment cannot be explained by the 5% antibiotic loss detected by HPLC. Another group also observed the diminished activity of ceftazidime against *E. coli* when media was enriched with glucose (Malouin *et al.*, 1991). They reported that the glucose concentration was directly proportional to PBP 8 (Penicillin Binding Protein) production by *E. coli*, while it was inversely proportional to the antimicrobial antibiotic activity. Ceftazidime exerts its bactericidal effect by inhibiting the enzymes responsible for cell-wall synthesis, especially PBP3, though it has low affinity to PBP8 (Malouin *et al.*, 1991; Clairoux *et al.*, 1992). It is possible that the augmented BPB8, an enzyme that is not efficiently degraded by ceftazidime, allows the bacteria to continue wall synthesis and scape from antibiotic intervention. Nevertheless, the mechanism has not been elucidated so far and other studies should be carried out in order to evaluate the impact of this phenomenon *in vivo*.

The combination of ceftazidime with aminophylline resulted in ceftazidime degradation, which initiated between 2 and 6 hours after mixture preparation and peaked at 24 hours, summing 25% total loss of ceftazidime. Aminophylline is composed by a teophylline-etilenodiamine complex that is stable at basic solutions (Ishiguro *et al.*, 1980), and remained stable when combined with the antibiotic. On the other hand, maximum ceftazidime stability is achieved within the pH range of 4.5 to 6.5 (Zhou *et al.*, 1995), while its hydrolysis is favored in an alkaline environment (Vilanova *et al.*, 1993). The basic pH of the resulting mixture may by itself explain the significant loss of antibiotic concentration. It is unlikely that both drugs reacted with each other, mainly because aminophylline maintained its physicochemical stability. Accordingly, another cephalosporin combined with the same bronchodilator showed no pharmacokinetic interactions between the two drugs (Szmygin, 1996). The drug mixture in glucose parenteral solution was considered by another group as stable within a 0-2 h range and unstable within a 6 to 24 h range; results which were reproduced by our HPLC experiments (El-Shaboury, 2007). However, the same drug mixture at 0 hour presented ceftazidime MIC increase of 15 and 3.9 fold for *E. coli* and *P. aeruginosa*, respectively. The hydrolysis of ceftazidime triggered by the basic pH was probably enhanced by the 37 °C maintained throughout the MIC tests (Farina *et al.*, 1999). Since the human body maintains the same temperature, it is likely that degradation continues after drug infusion.

The MIC test proved to be a valuable tool when evaluating antibiotics stability in non-standard conditions. The biological method makes it possible to detect antibiotic and vehicle changes in the presence of the target microorganisms and nutritional factors at body temperature. It is important to highlight that MIC was used in our experiments to evaluate the loss of antibiotic activity when compared to an initial solution, but not with a biological reference standard. Therefore, further work is required to include MIC as a routine test for stability, including antibiotic potency determination and method validation.

The previously reported incompatibility of ceftazidime and aminophylline was confirmed by our data. In addition, it was demonstrated that the combination is not stable even immediately after the preparation, due to loss of antimicrobial activity of ceftazidime. Based on these findings, it is not advisable to administer ceftazidime with aminophylline in the same container or by the same intravenous set. Results also suggest that glucose parenteral solution may not be the best choice for a ceftazidime vehicle, especially when a degradation process has already been triggered.

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