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Stability of frozen stock solutions of beta-lactam antibiotics, cephalosporins, tetracyclines and quinolones used in antibiotic residue screening and antibiotic susceptibility testing

Lieve Okerman *, Johan Van Hende, Lieven De Zutter

Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Belgium

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Abstract

The stability of frozen stock solutions of antibiotics belonging to three different families was evaluated using an agar diffusion test, with *Bacillus subtilis* as a test strain. Diameters of inhibition zones were measured at monthly intervals during 6 months, and the decline in active substance was calculated. Penicillin and amoxicilline lost nearly half of their potency, the cephalosporins ceftiofur and cefapirin one quarter, but ampicillin was more stable. The quinolones flumequine, enrofloxacin and marbofloxacin were relatively stable; the loss of activity was less than 10% after 6 months of preservation at −20 °C. This was also the case for doxycycline and chlortetracycline, while oxytetracycline and tetracycline lost about 25% of their potency. When used in microbiology, i.e. for residue testing or for determination of minimum inhibitory concentrations, a diminution of activity less than 25% will not be noticed. For these applications, the four tetracyclines and three quinolones tested can be kept for 6 months at −20 °C, while the beta-lactam antibiotics should be discarded after 3 months. Standard stock solutions of beta-lactam antibiotics and cephalosporins should preferably be used the same day when they are intended for quantitative residue analysis.
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1. Introduction

Solutions of antibiotic standards are used daily in all laboratories performing routine analyses for antibiotic residues. Microbiological screening tests such as the Four Plate Test (FPT) are controlled with paper disks impregnated with a prescribed concentration of an antibiotic [1]. Screening and confirmatory methods are validated with samples spiked with the analyte or analytes at the levels of interest [2–5]. Standard solutions of antibiotics are also needed in food and clinical microbiology. Antibiotics are added to selective media, and to media in antibiotic susceptibility testing, i.e. determinations of minimal inhibitory concentrations (MIC) of antibiotics for bacterial species and strains [6].

For residue analysis, working standard solutions are normally prepared in two or more steps. First, a concentrated stock solution is made that can be kept for a few days, weeks or months. The working solutions, dilutions of the stock solutions to the required concentrations, are often intended for immediate use and discarded thereafter. However, stock solutions of many antibiotics are not stable and cannot be kept for a long time. The standard operating procedure for the FPT for example suggests that the penicillin G stock solution should be discarded after 4 days at 4 °C [1]. Tetracyclines are not stable in acidic and alkaline conditions [7].

Preparation of stock solutions is time consuming. Weighing out a few milligrams of the antibiotic and taking care that it is completely dissolved in the precise amount of solvent or water should be done by skilled and trained personnel. Only a small amount of the stock solution is needed for the analyses and most of it is discarded. A longer preservation time is an advantage for the laboratory and for the environment, but it is not well known if antibiotic stock solutions are stable and how to preserve them.

Antibiotics can be detected with microbiological inhibition tests. There is a linear correlation between the absolute quantity

* Corresponding author. Tel.: +32 92647456; fax: +32 92647459.
E-mail address: godelieve.okerman@ugent.be (L. Okerman).

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of a given antibiotic and the surface of an inhibition zone produced by it on an agar layer seeded with a sensitive bacterial strain [8]. Thus, the stability of an antibiotic solution can be evaluated by measuring the inhibition zones at different times of preservation.

The present paper describes test results obtained with a medium at pH6 seeded with *Bacillus subtilis*, in the investigation on the stability of frozen stock solutions of three penicillins, two cephalosporins, four tetracyclines and three quinolones. This medium was chosen because it is one of the plates used in the FPT, and it detects a large range of antibiotics, including tetracyclines, beta-lactam antibiotics and quinolones [9]. Stock solutions were prepared in water or methanol, kept at −20 °C and the activity was measured monthly. The practical consequences of the findings are discussed.

2. Materials and methods

The following antibiotic standards were purchased from Sigma (St. Louis, MO, USA): the penicillins ampicillin (anhydrous, A-9393), penicillin G (sodium salt, PEN-NA) and amoxicillin (A-8523); the cephalosporin cephapirin (sodium salt, C-8270); the tetracyclines doxycycline (hyclate, D-9891), oxytetracycline (dihydrate O-5750); tetracycline (T-3258); chlortetracycline (hydrochloride, C-4881), and the quinolone flumequine (F-7016). The cephalosporin ceftiofur sodium was obtained from Pharmacia (Puurs, Belgium); the fluoroquinolones marbofloxacin from Vetoquinol (Magny-Vernois, France) and enrofloxacin from ICN Biomedical (Aurora, OH, USA).

The experiments were planned over a period of 6 months. Measurements were done for the first time after 3 months, then at monthly intervals, and each time compared with results obtained with dilutions of freshly prepared stock solutions. Stock solution A was prepared at time 0 and tested after 3–6 months. Stock solution B was prepared after 3 months, and tested at 3–6 months after the start of the experiments, thus when 0–3 months old. Stock solution C was prepared after 4 months, and tested at 4–6 months, thus when 0–2 months old. Stock solution D was prepared at 5 months and tested at 5 and 6 months, thus when 0 and 1 month old. Stock solution E was prepared at 6 months and tested immediately after preparation. Freshly prepared stocks were tested at four different occasions ($n = 80$), 1 month old solutions at three different occasions ($n = 60$), 2- and 3-month-old solutions at two different occasions ($n = 40$), 4–6-month-old solutions only once ($n = 20$).

Stock solutions of 1 mg mL^{-1} were prepared as follows: 10.0 ± 0.3 mg were weighed and dissolved in 10 ± 0.05 mL distilled water (penicillins and cephalosporins), methanol (tetracyclines) or 0.1N NaOH (quinolones). The stock solutions were divided into aliquots of approximately 1 mL in Eppendorf tubes and kept at −20 °C until needed. The tubes were stored in a paper box, and care was taken that they were never exposed to direct strong light.

Test agar pH6 (Merck, Darmstadt, Germany) was prepared, sterilised and cooled to 45 °C, and 100 μL of *B. subtilis* BGA spore suspension (Merck) were added to 100 mL of agar immediately before pouring 5.0 ± 0.1 mL in 90 mm petri dishes. The seeded plates were stored in airtight containers at 4 °C and used for analysis within 5 days. Just before analysis, four paper disks (diameter 12.7 mm, Schleicher und Schuell, Dassel, Germany) were laid upon the plates at a distance of approximately 1 cm from the edges.

The tests were done with freshly prepared dilutions of the stock solutions. For the stability study the stock solutions were diluted as follows: ampicillin 1/4000 (0.25 μg mL^{-1}); penicillin G 1/10,000 (0.10 μg mL^{-1}); amoxicillin 1/5000 (0.20 μg mL^{-1}); ceftiofur 1/1000 (1.0 μL mL^{-1}); cefapirin 1/5000 (0.20 μg mL^{-1}); doxycycline 1/5000 (0.20 μg mL^{-1}); oxytetracycline 1/2000 (0.50 μg mL^{-1}); tetracycline 1/2000 (0.50 μg mL^{-1}); chlortetracycline 1/5000 (0.20 μg mL^{-1}); marbofloxacin 1/2000 (0.50 μg mL^{-1}); enrofloxacin 1/2000 (0.50 μg mL^{-1}); flumequine 1/2000 (0.50 μg mL^{-1}). The 50 μL of the solutions were added to each of 20 paper disks A prediffusion time of 1 h at room temperature was used to avoid variability in time between application of standard solutions and start of the incubation. All plates were incubated overnight at 30 °C.

The diameters of the zones were measured with sliding callipers and the average and standard deviations of 20 observations were calculated for each measuring point. These average diameters were presented in diagrams, and the trendlines were calculated.

Finally the loss of activity was estimated by comparing the diameters at each measuring point with the calibration curves of the respective antibiotics. Calibration curves or dose–response curves were calculated by testing 2–4 two-fold dilutions of the antibiotics, prepared from a fresh stock solution. Four or more observations were obtained for each concentration tested. Plates with paper disks were prepared as described higher for the stability tests, and 50 μL of the two-fold dilutions were pipetted upon the paper disks. The concentration range was chosen around the concentration used for stability testing, in order to obtain zones ranging from 13 up to 30 mm. In this range, a linear correlation exists between the absolute quantity of active antibiotic and the surface of the zone, or between the logarithm of this quantity and the diameter of the zone. The plates were further treated and the zones were read as for the stability tests. The equation of the regression line of the zone diameters versus log(ng/disk) enabled to calculate remaining active substances at each point of the stability study.

3. Results

Intradays repeatability of the method is illustrated in Table 1. The variation between the average zone diameters when fresh stock solutions were used was moderate (between 1 and 2 mm) for ampicillin, amoxicillin and marbofloxacin, and low to very low for the other antibiotics.

Tables 2 and 3 represent the evolution of the average of 20 inhibition zones in function of the number of days after dissolution of the standards in water or methanol. The corresponding equations and the correlations between time of conservation and diameters of zones without growth are given in Table 2, while

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