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Cefotaxime Stability during In Vitro Microbiological Testing

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Cefotaxime is a broad-spectrum cephalosporin which is metabolized or degraded to less active or inactive metabolites by serum esterases, elevated temperatures, or a pH outside of its stability range. Cefotaxime instability during in vitro microbiological susceptibility tests may lead to an underestimation of the antibacterial activity of the compound. Cefotaxime and desacetylcefotaxime solutions were studied under MIC and serum inhibitory titer testing conditions. Cefotaxime concentrations, as measured by high-performance liquid chromatography, decreased 20 to 30% over the incubation period in various systems tested; the greatest decline occurred in systems containing serum in the media. Changes in the results of microbiological susceptibility tests interpreted after 6 and 18 h of incubation were consistent with changes observed in the high-performance liquid chromatography analysis. This study demonstrates cefotaxime instability under conditions of in vitro microbiological testing.

Cefotaxime (CTX) is a cephalosporin antibiotic with a broad spectrum of antibacterial activity known to be partially metabolized in vivo by esterases (3, 5, 7). The major metabolite, desacetylcefotaxime (des-CTX), possesses antibacterial activity, although less than that of the parent compound (1, 4, 8, 15). In vitro stability of CTX may be affected by physical factors such as pH and temperature (6, 7).

This known metabolism and degradation of CTX raises the question of the stability of the compound during in vitro microbiological testing. These tests involve the incubation of antibiotic solutions at 35 to 37°C over 18 to 24 h in various broth or serum-broth combination media. Of particular interest is the stability of CTX during a serum inhibitory titer-serum bactericidal titer (SIT-SBT) determination. The SIT test, first described by Schlichter et al. (13) and later modified by Reller and Stratton (12, 16), provides ideal conditions for CTX degradation for two reasons. First, by using serum as a component of the media, it simulates in vivo conditions which may lead to deacetylation of the compound by esterases. Second, it involves incubation of the drug for 18 to 24 h at 35 to 37°C, which may lead to further drug decomposition due to physical factors such as temperature and pH changes. This degradation may result in an underestimation of the in vivo activity of CTX, leading to a clinical decision to needlessly give higher doses of the drug, add another antimicrobial agent to the regimen, or even abandon therapy with the agent.

This study was undertaken to determine the stability of CTX and des-CTX under in vitro microbiological test conditions.

MATERIALS AND METHODS

CTX analytical grade powder (lot no. RP5008) containing 929 μg of CTX activity per 1,000 μg of powder and des-CTX analytical grade powder (lot no. RP5117) containing 1,000 μg of des-CTX per 1,000 μg of powder (Hoechst-Roussel Pharmaceuticals) were used in all phases of the study. Serum was obtained from a single, healthy volunteer to minimize potential variances in serum composition. Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.; lot no. 737928) supplemented with 50 mg of calcium per liter and 25 mg of magnesium per liter (MHB-S) was utilized.

CTX and des-CTX concentrations were determined by high-performance liquid chromatography with Waters Associates (Milford, Mass.) chromatography equipment. Samples were analyzed by the assay of Yost and Derendorf (19) with a mobile phase of 0.007 M phosphoric acid in water-acetonitrile (85:15) at a flow rate of 1.3 ml/min. All assays were performed at ambient conditions at A_{309} with retention times for CTX and des-CTX of 10 and 4 min, respectively. Samples of 1 ml were vortexed with 3.0 ml of acetonitrile and then centrifuged for 20 min; 1.0 ml of the resulting supernatant was extracted with 1.0 ml of chloroform–1-butanol (3:1) and was vortexed for 10 s. After a 5-min centrifugation, 20 μl of the resultant supernatant was injected into the high-performance liquid chromatography system. Concentrations were calculated by linear regression from standard curves that utilized a concentration range of 0.5 to 25 μg/ml.

CTX and des-CTX stability under various in vitro microbiological susceptibility test conditions. (i) Stability under SIT test conditions. Five different solutions composed of 50% serum and 50% MHB-S were prepared to simulate SIT tests. Test solutions contained 10 μg of CTX or des-CTX per ml, alone or in combination. In an effort to determine whether the presence of microorganisms affected the disposition of the compounds, an inoculum of approximately 5×10^5 CFU of *Escherichia coli* (ATCC 25922) per ml was added to one solution, and the same inoculum of *Proteus vulgaris* (ATCC 13315) was added to another.

A 10-ml sample of each solution was prepared. Immediately after preparation (time 0), a 1.0-ml portion was removed and assayed for CTX or des-CTX concentration or both. The remaining solution was incubated at 37°C. At 3-h intervals over the next 18 h, 1.0-ml samples were removed and analyzed for CTX or des-CTX concentration or both. Samples were assayed in duplicate, and the mean concen-

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TABLE 1. CTX concentration at selected times

Prepn	CTX concn (μg/ml) at:			
	0 h	6 h	12 h	18 h
CTX in broth-serum	9.89	9.36	8.00	6.82 (69.0)[a]
CTX–des-CTX in broth-serum	10.29	9.70	7.72	7.38 (71.7)
CTX–des-CTX in broth-serum with *E. coli*	9.91	9.02	7.63	6.75 (68.1)
CTX–des-CTX in broth-serum with *P. vulgaris*	10.01	9.17	7.69	6.23 (62.2)
CTX–des-CTX in broth	10.52	10.14	9.06	8.49 (80.7)

[a] Numbers within parentheses indicate percentages of time zero concentrations.

tration obtained at each time point was reported. The pH of each sample was measured at each time point.

(ii) Stability under MIC test conditions. Solutions containing 10 μg of CTX and 10 μg of des-CTX per ml were prepared in MHB-S alone to simulate MIC test conditions. This solution was then sampled, incubated, and analyzed as described above.

Microbiological susceptibility testing. To further investigate the effects of CTX and des-CTX degradation during in vitro microbiological testing, actual microbiological tests were performed. *E. coli* (ATCC 25922) and *P. vulgaris* (ATCC 13315) were used for all tests. All tests were performed in triplicate, and modal results are reported.

(i) MIC-MBC determination. The MIC and MBC were determined by microdilution procedures as previously described (9, 17) with a semiautomated diluter (Dynatech Laboratories, Inc., Alexandria, Va.). CTX and des-CTX concentrations ranged from 0.008 to 16 μg/ml with an inoculum of approximately 5×10^5 CFU/ml. After incubation periods of 6 to 18 h the MIC, defined as the lowest antibiotic concentration to inhibit visible growth of the organism, was determined. The MBC, defined as the lowest concentration of antibiotic that killed 99.9% of the initial inoculum after a 24-h incubation, was determined by subculturing all wells that inhibited visible growth onto Mueller-Hinton agar (Difco).

(ii) SIT-SBT determination. SIT-SBT determinations were performed by microtiter procedures as previously described (12, 14, 16) with initial spiked CTX and des-CTX concentrations of 16 μg/ml. Each well contained 100 μl of 1:1 serum–MHB-S. After 6 and 18 h of incubation at 37°C the SITs were determined. Wells that did not show visible growth after 18 h of incubation were subcultured, and the SBTs (99.9% kill) were determined.

(iii) Checkerboard synergy testing. Antibiotic synergy studies between CTX and des-CTX were performed in triplicate by the checkerboard method (2, 10, 11). CTX and des-CTX concentrations ranged from three times the MIC to four dilutions below the MIC for each agent. The test was read after 6 and 18 h of incubation at 37°C. Results of the synergy studies were interpreted by calculation of the fractional inhibitory concentration as follows: $(MIC_{c\text{-}c}/MIC_{c\text{-}a}) + (MIC_{d\text{-}c}/MIC_{d\text{-}a})$, where $MIC_{c\text{-}c}$ is the MIC of CTX in combination, $MIC_{c\text{-}a}$ is the MIC of CTX alone, $MIC_{d\text{-}c}$ is the MIC of des-CTX in combination, and $MIC_{d\text{-}a}$ is the MIC of des-CTX alone.

RESULTS

CTX and des-CTX stability under various in vitro microbiological susceptibility test conditions. Standard curves of CTX and des-CTX were prepared daily from a previously prepared stock solution stored at −70°C. The within-day coefficients of variation were 3.75 and 11.7% for CTX and des-CTX, respectively. The between-day coefficients of variation for CTX and des-CTX were 3.1 and 7.2%, respectively.

Table 1 shows the concentration of CTX obtained after various incubation times under various conditions. CTX concentration decreased over the incubation period in all systems tested. In the systems containing serum the loss of CTX was approximately 30%. This loss was consistent, regardless of the presence of des-CTX or microorganisms in the media. The loss of CTX over time was approximately 20% when incubated in broth alone.

The study revealed a less dramatic loss of des-CTX when compared with CTX (Table 2). The decrease in des-CTX concentration when incubated alone in media containing serum was approximately 20%. When des-CTX was incubated in combination with CTX, the apparent loss of des-CTX was very small or even appeared to increased slightly. The concentration of des-CTX was unaffected by the presence of microorganisms.

The pH of the solutions ranged from 7.2 to 8.5. There was no observed trend in the pH in any of the systems tested.

Microbiological susceptibility testing. (i) MIC-MBC determination. MIC and MBC results after 6 and 18 h of incubation are presented in Table 3. The MICs obtained are within the expected range for the ATCC strains tested.

(ii) SIT-SBT determination. Inhibitory titers and bactericidal titers obtained are presented in Table 3. The *P. vulgaris* strain was serum sensitive; therefore only the results of the SIT-SBT test with *E. coli* are presented.

(iii) Antibiotic synergy testing. The calculated fractional inhibitory concentration for the system tested was 0.75. Therefore, neither synergy nor antagonism was found between CTX and des-CTX for the strain of *E. coli* tested.

DISCUSSION

In vitro susceptibility testing of antimicrobial agents plays a major role in predicting the efficacy of an antibiotic against clinical pathogens. The choice and dose of antimicrobial therapy today are often guided by the MIC or SIT of an antibiotic for a particular bacterial strain. If an antimicrobial agent is not stable under the in vitro testing conditions and is metabolized or degraded to less active or inactive compounds, the in vivo efficacy of the agent may be underestimated.

The results of our study with CTX and des-CTX clearly demonstrate the instability of these compounds during in vitro broth dilution susceptibility and SIT-SBT testing. This

TABLE 2. des-CTX concentration at selected times

Prepn	des-CTX concn (μg/ml) at:			
	0 h	6 h	12 h	18 h
des-CTX in broth-serum	11.39	9.90	9.76	9.27 (81.4)[a]
CTX–des-CTX in broth-serum	11.21	12.02	11.87	11.49 (102.5)
CTX–des-CTX in broth-serum with *E. coli*	10.90	10.79	10.97	10.64 (97.6)
CTX–des-CTX in broth-serum with *P. vulgaris*	10.80	10.91	10.89	11.08 (102.6)
CTX–des-CTX in broth	9.89	10.43	9.11	9.56 (96.7)

[a] Numbers within parentheses indicate percentages of time zero concentrations.

TABLE 3. MIC, MBC, SIT, and SBT of CTX and des-CTX after 6 and 18 h

Micro-organism	Time (h)	CTX				des-CTX			
		MIC (μg/ml)	MBC (μg/ml)	SIT	SBT	MIC (μg/ml)	MBC (μg/ml)	SIT	SBT
E. coli	6	0.125	ND[a]	1:512	ND	1.0	ND	1:32	ND
	18	0.125	0.125	1:256	1:256	1.0	1.0	1:16	1:16
P. vulgaris	6	<0.008	ND	ND	ND	<0.008	ND	ND	ND
	18	1.0	2.0	ND	ND	8.0	16.0	ND	ND

[a] ND, Not done.

instability is evidenced by a decrease in the concentration of the compounds over the 18-h incubation period required by these tests. These results are consistent with the earlier work of Wick (18) with cephalothin, a cephalosporin which is also metabolized to a desacetyl metabolite. This loss of drug may be attributed to metabolism by esterases in the serum resulting in deacetylation of the compound as well as degradation by exposure to the incubation conditions. This effect appears unrelated to action of microorganisms in the system.

The metabolite concentration underwent little change over time when it was incubated in combination with the parent compound. This apparent stability of des-CTX could be explained by formation of the metabolite during incubation rather than an interaction with the parent compound.

An attempt was made to model the rate of loss of CTX and des-CTX. However, the 18-h sampling period was too short relative to the rate of reaction; therefore, it was not possible to discern whether the process represented zero-order or first-order loss.

Microbiological susceptibility tests also revealed a decrease in CTX and des-CTX activity over time. In the MIC determination for *P. vulgaris*, there was a marked difference between the results read after 6 and 18 h of incubation. At 6 h, both CTX and des-CTX inhibited the growth of the organisms at all concentrations tested, whereas after 18 h it appeared that higher concentrations of both compounds were required to inhibit growth. It should be noted that the control wells showed growth after 6 h of incubation, suggesting that the results obtained were not due to poor growth of the organism. Perhaps the differences noted at 6 and 18 h can be attributed to loss of activity of the compounds due to metabolism or degradation. The same pattern was not seen with the *E. coli*, since the MIC at 6 h was the same as that at 18 h. This finding cannot be readily explained, since we found similar decreases in drug concentrations in both *E. coli* and *P. vulgaris* systems in the high-performance liquid chromatography portion of this study.

The SIT determination with *E. coli* also point to a diminution of CTX and des-CTX activity over the incubation period. The titers at 6 h were 1:512 and 1:32 for CTX and des-CTX, respectively. After 18 h of incubation, however, the respective titers were 1:256 and 1:16, indicating that CTX and des-CTX activities were decreased. It should be noted that the serum used was not heat inactivated and therefore probably contributed to inhibition of growth of the organism.

Metabolism and degradation of antimicrobial agents during common in vitro susceptibility testing may result in an underestimation of actual in vivo activity. We found a substantial decrease in cefotaxime concentrations under conditions simulating these tests. Results from actual in vitro microbiological tests seem to confirm the results obtained from these simulated tests. Losses of the magnitude found could lead to inappropriate conclusions regarding MIC determinations and serum inhibitory and bactericidal activity. Other antibiotics with known or suspected stability problems or those known to be metabolized by serum esterases may also undergo significant metabolism or degradation or both under these conditions. The extent of antimicrobial degradation during in vitro microbiological testing has not been well studied, and further work is needed to define the extent of this problem.

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LITERATURE CITED

1. **Aldridge, K. E., C. V. Sanders, and R. L. Marier.** 1984. In vitro synergy and potentiation between cefotaxime and desacetylcefotaxime against clinical isolates of *Bacteroides*. Diagn. Microbiol. Infect. Dis. **2**:47S–53S.
2. **Berenbaum, M. C.** 1978. A method for testing for synergy with any number of agents. Infect. Dis. **137**:122–130.
3. **Chamberlain, J., J. D. Coombes, D. Dell, J. M. Fromson, R. J. Ings, C. M. Macdonald, and J. McEwen.** 1980. Metabolism of cefotaxime in animals and man. Antimicrob. Chemother. **6**(Suppl. A):69–78.
4. **Chin, N., and H. C. Neu.** 1984. Cefotaxime and desacetylcefotaxime: an example of advantageous antimicrobial metabolism. Diagn. Microbiol. Infect. Dis. **2**:21S–31S.
5. **Coombes, J. D.** 1982. Metabolism of cefotaxime in animals and humans. Rev. Infect. Dis. **4**:S325–S332.
6. **Das Gupta, V.** 1984. Stability of cefotaxime sodium as determined by high-performance liquid chromatography. Pharm. Sci. **73**:565–567.
7. **Fabre, H., N. H. Eddine, and G. Berge.** 1984. Degradation kinetics in aqueous solution of cefotaxime sodium, a third-generation cephalosporin. Pharm. Sci. **73**:611–618.
8. **Jones, R. N., A. L. Barry, T. L. Gavin, and J. A. Washington II.** 1985. Susceptibility tests: Microdilution and macrodilution broth procedures, p. 972–977. *In* E. H. Lennette, A. Balows, W. J. Hausler, and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
9. **Jones, R. N., A. L. Barry, and R. R. Packer.** 1984. The activity of cefotaxime and desacetylcefotaxime alone and in combination against anaerobes and staphylococci. Diagn. Microbiol. Infect. Dis. **2**:37S–46S.
10. **Krogstad, D. J., and R. C. Moellering, Jr.** 1986. Antimicrobial combinations, p. 537–595. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 2nd ed. The Williams & Wilkins Co., Baltimore.
11. **Marymont, J. H., Jr., and J. Marymont.** 1981. Laboratory evaluation of antibiotic combinations: a review of methods and problems. Lab. Med. **12**:47–55.
12. **Reller, L. B., and C. W. Stratton.** 1977. Serum dilution test for bactericidal activity. II. Standardization and correlation with antimicrobial assays and susceptibility tests. Infect. Dis. **136**:196–204.
13. **Schlichter, J. G., H. MacLean, and A. Milzer.** 1949. Effective penicillin therapy in subacute bacterial endocarditis and other chronic infections. Am. J. Med. Sci. **217**:600–608.
14. **Schoenknecht, F. D., L. D. Sabath, and C. Thornsberry.** 1985. Susceptibility tests: special tests, p. 1000–1008. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
15. **Schrinner, E., M. Limbert, K. Seeger, G. Seibert, and W. J. Novick, Jr.** 1984. The in vitro antimicrobial activity of desacetylcefotaxime compared to other related beta-lactams.

Diagn. Microbiol. Infect. Dis. **2**:13S–20S.
16. **Stratton, C. W., and L. B. Reller.** 1977. Serum dilution test for bactericidal activity. I. Selection of a physiologic diluent. Infect. Dis. **136**:187–195.
17. **Thrupp, L. D.** 1986. Susceptibility testing of antibiotics in liquid media, p. 93–150. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 2nd ed. The Williams & Wilkins Co., Baltimore.
18. **Wick, W. E.** 1964. Influence of antibiotic stability on the results of in vitro testing procedures. J. Bacteriol. **87**:1162–1170.
19. **Yost, R. L., and H. Derendorf.** 1985. Rapid chromatographic determination of cefotaxime and its metabolite in biological fluids. Chromatography **341**:131–138.