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Solvent degradation of cloxacillin *in vitro*

Tentative identification of degradation products using thermospray liquid chromatography–mass spectrometry

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ABSTRACT

The determination of cloxacillin and the other penicillin antibiotics at trace levels in pharmaceutical samples and biological fluids and tissues is difficult. Degradation of penicillins occurs during exposure to chemicals and solvents used in sample extraction, storage of extracts and chemical analysis. One of the most important factors for the determination of cloxacillin in bovine milk and tissues is the choice of solvent used for extraction. Cloxacillin stored in different solutions underwent two types of degradation: hydrolysis with decarboxylation to yield cloxacillinpenilloic acids when water or aqueous solutions of acetonitrile or 2-propanol were used, and alcoholysis to form cloxacillinpenicilloic acid ester when methanol or ethanol was used. Cloxacillin stored in aqueous methanol or acetonitrile solution underwent faster degradation when stored in water or aqueous solutions of 2-propanol or ethanol. The solvent combinations that resulted in minimum sample degradation of cloxacillin were acetonitrile–ethanol–water (25:25:50) or ethanol–water (50:50). Degradation of cloxacillin was faster at the lower levels (500 and 50 ng/ml) of the antibiotic for all solvents tested. Nearly complete degradation of cloxacillin was observed at the 50 ng/ml level after 2 h at 20°C. Degradation was nearly non-existent at the 1 mg/ml level of the antibiotic in each solvent.

INTRODUCTION

The determination of β-lactam antibiotics in pharmaceutical samples, biological fluids or tissues is difficult owing to solvent degradation occurring during sample extraction, storage and chemical analysis. Degradation of β-lactam in the presence of methanol has been reported [1] and solvents used in sample preparation or storage of extracted samples could account for some degradation products reported for penicillins [2–13]. Degradation in common solvents used in β-lactam analysis, such as methanol or acetonitrile [14–20], could prevent their determination at part per billion (ppb) levels. Further, under these circumstances, the determination of penicillins at ppb levels may be based on the

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detection of degradation products with nearly identical retention times in the absence of a specific liquid chromatographic (LC) detector. In order to achieve ppb determinations of β-lactams, the stability of these antibiotics in various solvents used for sample extraction from milk or tissue and LC analysis must be studied. This paper reports the extent of degradation observed for cloxacillin in various solvents and identified the major degradation products formed.

EXPERIMENTAL

Materials and reagents

A 100 μg/ml cloxacillin (Sigma, St. Louis, MO, USA) solution was prepared in the following solvents: acetonitrile–water (50:50), acetonitrile, methanol–water (50:50), methanol, water, ethanol–water (50:50), 2-propanol–water (50:50), acetonitrile–0.2% aqueous phosphoric acid (50:50), acetonitrile–acetic acid–0.1 *M* aqueous ammonium acetate (50:2:48) and acetonitrile–ethanol–water.

Acetonitrile, methanol, ethanol and 2-propanol were of high-performance liquid chromatographic (HPLC) grade (American Burdick and Jackson, Muskegon, MI, USA), water was obtained from Hydro Services and Supplies (Research Triangle Park, NC, USA) and ammonium acetate was of HPLC grade (Fisher Scientific, Raleigh, NC, USA). The cloxacillin solutions were stored in amber-glass vials for up to 8 weeks at −20°C. Every 1–2 weeks the samples were analyzed by liquid chromatography–ultraviolet detection (LC–UV). The sample solutions of cloxacillin were analyzed by thermospray LC–mass spectrometry (MS) at the 4–8-week point in the stability study to identify degradation products formed.

The correlation between degradation rate and concentration of cloxacillin was also investigated from the 1 mg/ml–50 ng/ml levels. Solutions of 1000, 100 and 0.5 μg/ml and 50 ng/ml of cloxacillin in acetonitrile–water (50:50), methanol–water (50:50), ethanol–water (50:50), isopropanol–water (50:50) and water were stored in amber-glass vials for three weeks at −20 or 20°C (room temperature). The stored samples were analyzed weekly by LC–UV to determine the extent of degradation.

Liquid chromatography–ultraviolet detection

The LC–UV equipment consisted of a Waters Model W 600 solvent-delivery system coupled to a Model 900 UV–VIS photodiode-array detector (Waters Chromatography Division, Milford, MA, USA). The LC separation was conducted using a mobile phase consisting of acetonitrile–water (30:70, v/v) containing 5 m*M* dodecanesulfonate (Regis, Morton Grove, IL, USA) and 0.1% (v/v) 85% phosphoric acid at a flow-rate of 0.5–0.7 ml/min. The separation of the degradation product was accomplished on an Ultremex phenyl 3-μm analytical column 150 × 4.6 mm I.D.) (Phenomenex, Torrance, CA, USA). The UV–VIS photodiode-array detector was set to a wavelength range of 200–310 nm at 0.01–0.3 a.u.f.s.

Liquid chromatography–mass spectrometry

Thermospray LC–MS was applied under the following conditions. The LC mobile phase was isopropanol–acetic acid–0.25 *M* aqueous ammonium acetate solution (12:2.5:85.5) at a flow-rate of 1 ml/min and an Ultremex phenyl 3-μm analytical column (150 × 4.6 mm I.D.) was used for the separation. The thermospray interface (Finnigan MAT, San Jose, CA, USA) was operated with source and vaporizer temperatures of 300 and 130°C, respectively. The thermospray interface was mounted on a Finnigan MAT 4800 quadrupole mass spectrometer. The mass spectrometer was operated in the positive-ion detection mode, scanning from 150 to 600 u in 2 s.

RESULTS AND DISCUSSION

The major aim of this study was to determine the degradation products formed when cloxacillin was exposed to solvents commonly used in sample preparation. To verify the purity of cloxacillin and demonstrate that no artifact signals originating from the analysis method were present, a newly prepared aqueous solution of cloxacillin (structure I, Fig. 1) was analyzed by thermospray LC–MS. This analysis indicated that no impurities were present in the standard and no degradation products were formed in the LC–MS analysis (Fig. 2). The thermospray mass spectrum of this standard exhibited an $[M + H]^+$ ion at *m/z* 436 and several protonated fragment ions at *m/z* 410, 277 and 160. All protonated frag-

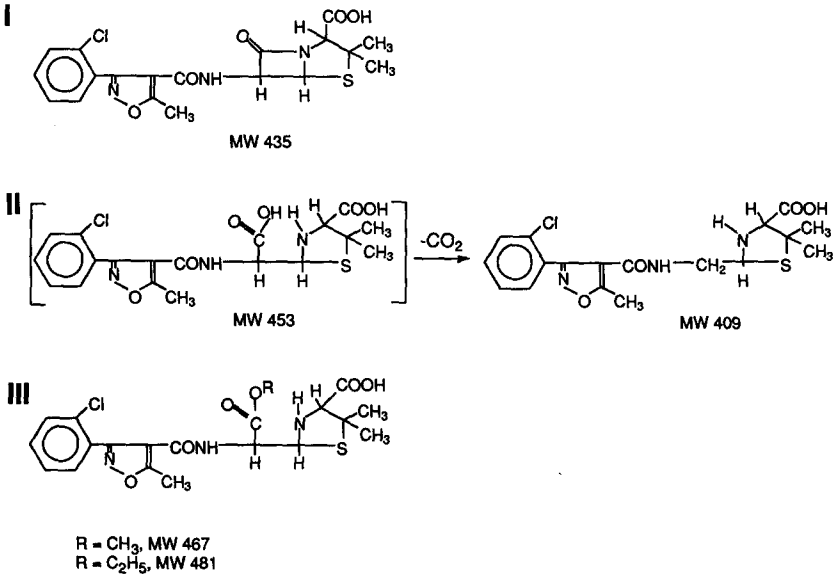

Fig. 1. Structures of (I) cloxacillin, (II) cloxacillinpenicilloic acid (left) and cloxacillinpenilloic acid (right) and (III) cloxacillinpenicilloic acid methyl and ethyl esters. MW = Molecular weight.

ment ions of cloxacillin involve ring opening and cleavage of the β-lactam ring. The ion at m/z 160 is representative of all penicillin antibiotics and the ion at m/z 410 formed as a result of thermal degradation of cloxacillin [21].

The storage of cloxacillin in water or in acetonitrile–water (50:50) for 2–4 weeks at −20°C resulted in sample degradation. LC–UV indicated the presence of three components in acetonitrile–water (50:50) (Fig. 3). Water resulted in a similar chromatogram, showing only differences in the relative intensities of the three peaks. The first peak (1) produced the thermospray mass spectrum shown in Fig. 4. This spectrum was interpreted as being clox-

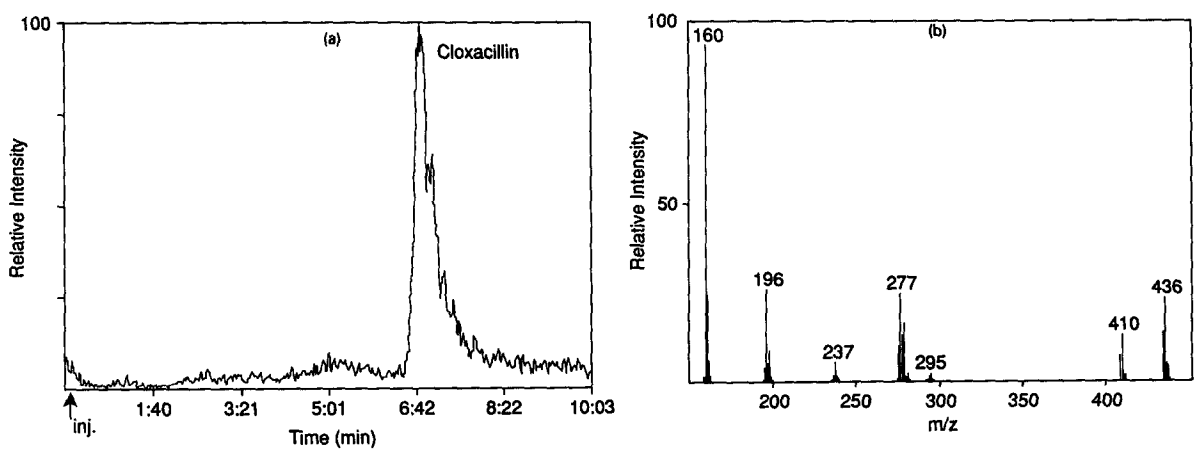

Fig. 2. Analysis of a newly prepared cloxacillin in water solution by thermospray LC–MS. (a) Chromatogram; (b) mass spectrum of cloxacillin.

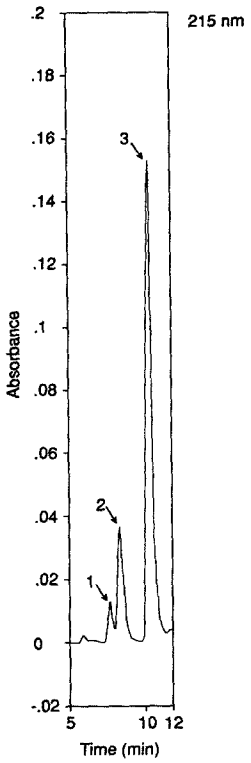

Fig. 3. LC–UV of cloxacillin (100 μg/ml) after 2 weeks in acetonitrile–water (50:50). Peak 1 is the β-epimer, peak 2 is the α-epimer of cloxacillinpenilloic acid and peak 3 is cloxacillin.

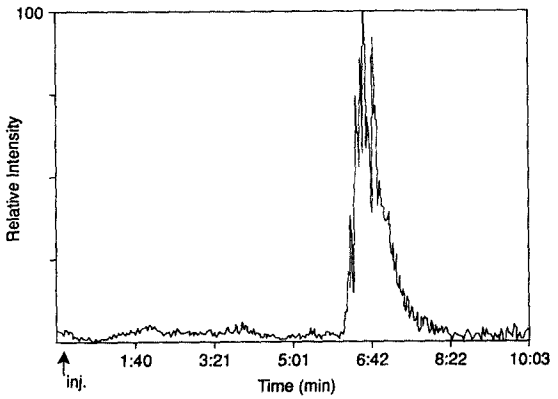

Fig. 5. Thermospray LC–MS chromatogram obtained after 4 weeks of storage of cloxacillin in methanol–water (50:50). This resulted in 100% conversion into its methyl ester.

acillin-β-penilloic acid (structure II, right, Fig. 1). The spectrum exhibited an $[M + H]^+$ ion at m/z 410 and several protonated fragment ions at m/z 279, 237 and 196. As the β-lactam ring was opened in the formation of the cloxacillin-β-penilloic acid, the ion at m/z 160 (characteristic of β-lactams) was absent. The second peak (2) in the chromatogram exhibited the identical thermospray mass spectrum to Fig. 4 and showed the same UV spectral curve as the β-penilloic acid. Based on this information it was postulated that these two peaks were epimers (cloxacillin-α- and β-penilloic acids). Further investigation showed that peak 1 was converted into peak 2 with time in both solutions. This conversion

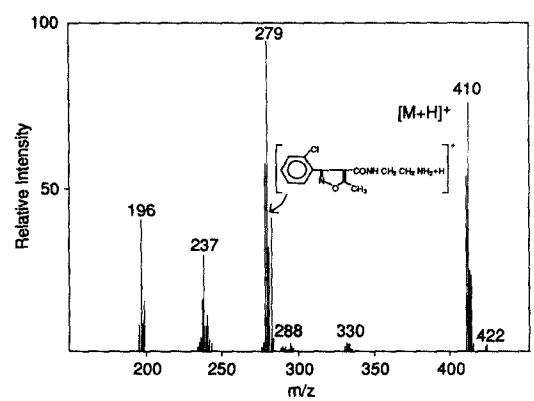

Fig. 4. Thermospray LC–mass spectrum of cloxacillin-β-penilloic acid in acetonitrile–water (50:50). The spectrum for the compound in pure water was identical.

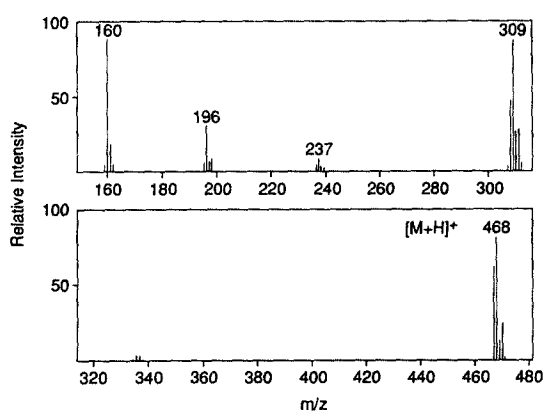

Fig. 6. Thermospray LC–mass spectrum of cloxacillinpenicilloic acid methyl ester stored in methanol–water (50:50) for 2 weeks.

was consistent with a report in the literature that indicated cloxacillin-β-penilloic acid is converted into cloxacillin-α-penilloic acid [3]. The last peak in the chromatogram (3) was cloxacillin. This peak showed a thermospray mass spectrum that was identical with that in Fig. 2b for cloxacillin in both solutions.

The storage of cloxacillin in methanol solution resulted in the formation of different degradation products. Thermospray LC–MS analysis of cloxacillin stored in methanol for 4 weeks exhibited only one peak, the retention time of which was slightly longer than that of cloxacillin (Fig. 5). This peak was identified as the methyl ester of cloxacillin (structure III, Fig. 1). The thermospray mass spectrum of the peak (Fig. 6) showed an $[M + H]^+$ ion at m/z 468 and protonated fragment ions at m/z 309, 196 and 160. The fragmentation of the methyl ester is similar to that for cloxacillin. The ion at m/z 160 results from fragmentation after methanolysis instead of thermal opening of the ring. The ion at m/z 309 is the methyl ester of the ion observed at m/z 277 for cloxacillin.

The use of other common longer chain alcohols such as ethanol and 2-propanol also exhibited degradation of cloxacillin. The storage of cloxacillin in ethanol resulted in the formation of the cloxacillin ethyl ester (Fig. 7). The thermospray LC–MS mass spectrum exhibited an $[M + H]^+$ ion at m/z 482 and two characteristic protonated fragment ions at m/z 323 and 160. The use of 2-propanol did not result in formation of a propyl ester, but rather the cloxacillin-α- and β-penilloic acid epimers were detected. The thermospray mass spectrum of the α-epimer exhibited a spectrum nearly identical with the β-epimer spectrum shown in Fig. 4.

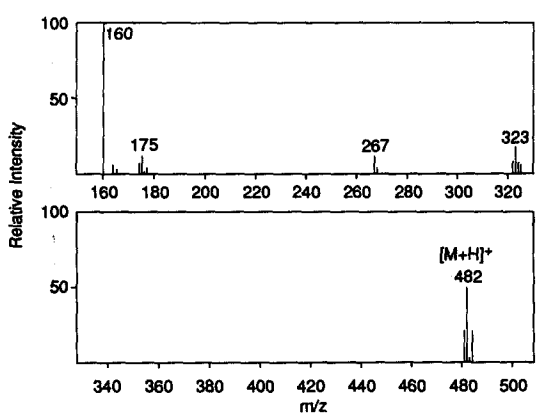

Fig. 7. Thermospray LC–mass spectrum of cloxacillinpenicilloic acid ethyl ester from a 100 μg/ml solution of cloxacillin stored in ethanol–water (50:50) for 6 weeks.

The storage of cloxacillin (100 μg/ml) in various solutions (Fig. 8) indicated that there was a wide range of degradation rates. Methanol resulted in the most rapid degradation of this antibiotic whereas acetonitrile–ethanol–water (25:25:50) resulted in the least degradation. After 2 weeks of storage in methanol–water (50:50) or methanol, cloxacillin was entirely converted into its methyl ester. Ethanol–water (50:50) resulted in a slower alcoholysis of cloxacillin compared with methanol. When cloxacillin was stored in 2-propanol–water (50:50), degradation initially occurred quickly, then slowed significantly over the 8-week study.

Cloxacillin underwent slower hydrolysis in water than in many organic or organic–water solutions. After 4 weeks of storage in water only 14% of cloxacillin had been converted into its degradation products. The storage of cloxacillin in acetonitrile for 4 weeks resulted in 25% degradation. The addition of 0.2 % of phosphoric acid or 2% of acetic acid in 0.1 *M* ammonium acetate (mobile phase for LC–UV or thermospray LC–MS) further increased the degradation rate. It was interesting that after 2 weeks further hydrolysis was not detected for the solutions containing phosphoric acid or acetic acid and ammonium acetate. Ethanol–acetonitrile–water (25:25:50) showed the least degradation, with only a 5% loss of cloxacillin after 4 weeks.

Most important, the degradation rate of cloxacillin was dependent on concentration. Lower concentrations of cloxacillin underwent faster degradation than higher concentrations. The storage of 0.5 μg/ml cloxacillin in methanol-water (50:50), 2-propanol–water (50:50), acetonitrile–water (50:50) and ethanol–water (50:50) resulted in 100%, 87%, 47% and 73% degradation, respectively of the antibiotic after 1 week. Cloxacilin completely degraded in the 0.5 μg/ml solution after 3 weeks of storage. Cloxacillin solutions stored in water resulted in 78% degradation in 1 week and 82% degradation in 3 weeks. The degradation rates for the five solutions evaluated at 0.5 μg/ml were significantly higher than those at the 100 μg/ml level as shown in Fig. 8.

Degradation of cloxacillin at the 50 ppb level occurred in a matter of hours at room temperature or

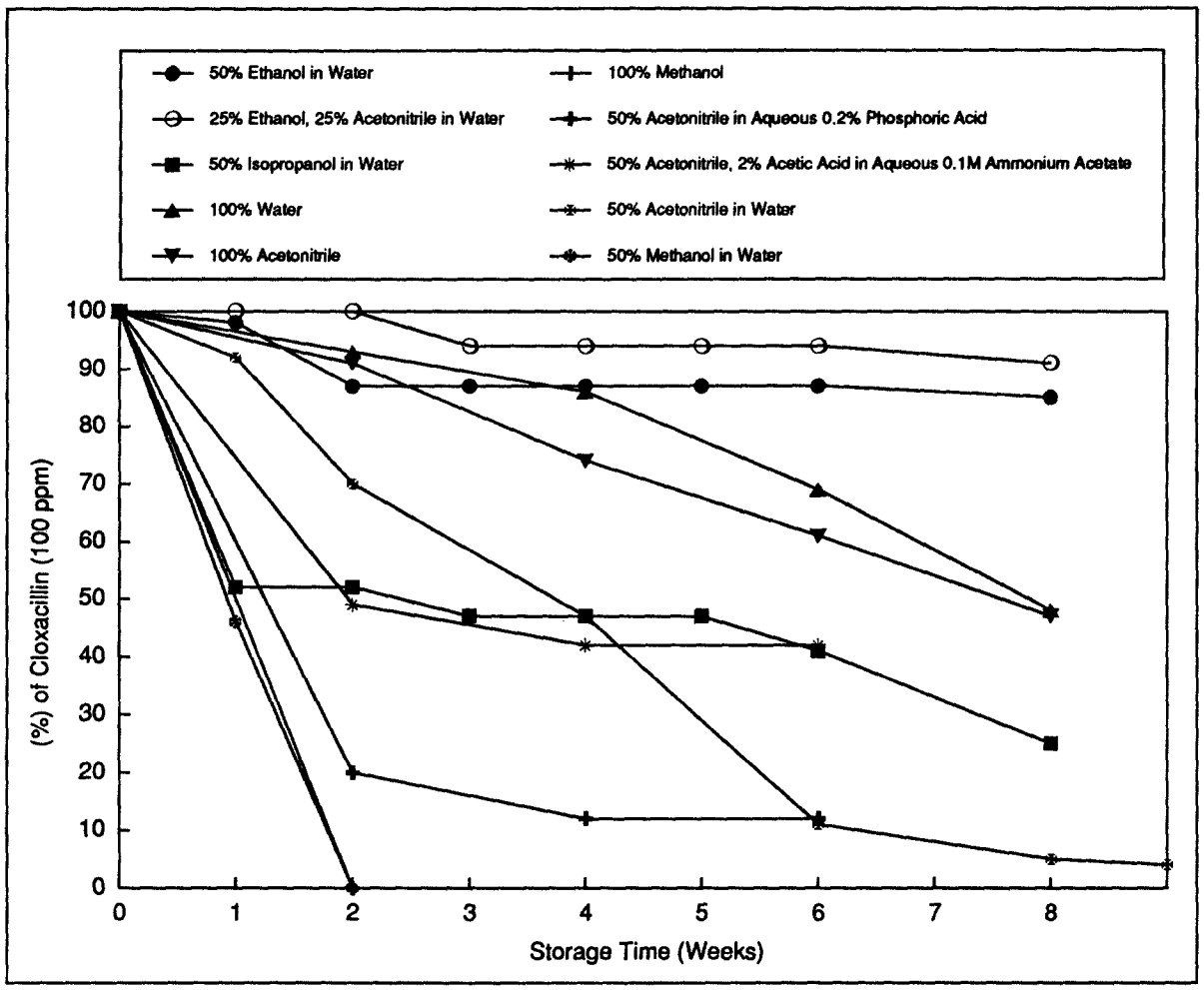

Fig. 8. Degradation of a 100 μg/ml solution of cloxacillin in different solvents during storage for up to 8 weeks at −20°C. This simulates the storage of sample extracts before LC analysis.

at −20°C. After storage of 50 ng/ml of cloxacillin in any organic-containing solvent for 2 h resulted in complete degradation. Degradation was slightly less at lower temperature (−20°C). It is obvious that these fast degradation rates can drastically alter the measurement of penicillin during extraction, storage of the extract before analysis and even during LC analysis. Water resulted in the least degradation for cloxacillin at the 50 ng/ml level, resulting in only about 18% degradation in 1 day and complete degradation within 3 weeks. On the other hand, cloxacillin stored in the same solvents at the 1000 μg/ml level did not undergo detectable degradation through the 3-week stability evaluation.

CONCLUSIONS

Degradation of trace (low ppb) levels of penicillins can occur in a matter of hours. Exposure of penicilins to organic solvents that are necessary for extraction or chromatography can lead to the formation of degradation products. Storage of a penicillin extract for analysis at −20°C in an organic-containing solvent used for extraction can result in complete degradation in as little as 2–4 h (for methanol). Cloxacillin stored in various solutions underwent two types of degradation: hydrolysis with decarboxylation in the presence of water, acetonitrile and 2-propanol and alcoholysis when methanol or

ethanol was present. As the methanolysis products form at a faster rate, methanol cannot be used in any step of an analytical procedure. With strict control of the analytical procedure, ethanol–water (50:50) or acetonitrile–ethanol–water (25:25:50) can be utilized for extraction from biological fluids or tissue and for the chromatographic separation of penicillins. If the extracts cannot be analyzed immediately, the samples should be stored dry or only in the presence of water at −20°C or lower temperatures to minimize degradation. Further, care must be taken in the determination of penicillins by LC in the absence of specific detectors, as some of the degradation products have very similar retention times to the undegraded antibiotic, possibly leading to misidentification.

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