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Investigation of a new amoxicillin sodium impurity unstable in solution

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Abstract

A new amoxicillin sodium impurity was detected by reversed-phase HPLC in commercial injectable preparations only when examined very soon after the drug was dissolved in the solvent vial (within about 10 min). The stability of this impurity was investigated by the degradation kinetic of its aqueous solutions. Ionspray mass spectrometry with flow-injection analysis and HPLC–MS methods were used to establish its nature. Some hypotheses concerning its chemical structure were formulated. The most likely assumption referred to the (5*S*,6*R*) amoxicillin piperazinedione diasteroisomer. The presence of the amoxicilloic acid methyl ester, an intermediate of the amoxicillin degradation process, was also hypothesized.

Keywords: Amoxicillin sodium; HPLC–MS; Ionspray mass spectrometry; Purity; Reversed-phase HPLC

1. Introduction

Amoxicillin and its sodium salt are among the most widely used β-lactam antibiotics because of their broad spectrum of antimicrobial activity and their low toxicity. Nevertheless, it is known that such compounds may be capable of inducing allergic reactions in sensitized individuals [1–3].

A number of papers have described specific HPLC methods for the determination of impurities derived from the synthesis or degradation of penicillins [4–9]. Moreover, structural investigations and kinetic studies have been performed as regards the formation of potentially antigenic compounds such as oligomers, penicilloyl esters and penicilloyl amides [10–14].

In the course of HPLC analyses on the purity determination of commercial pharmaceutical injectable preparations of amoxicillin sodium, an unknown impurity ("x") was found whose presence could be detected only within a very short time period after the preparation of the sample solution. The impurity had an appreciable UV absorption and was completely separated from the other known impurities and degradation products. This phenomenon was well observed in some

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commercially available pharmaceutical preparations which are no longer obtainable and to a less extent in other preparations from different sources, which are now on the market. All the preparations were examined during their validity period.

The aim of this work was to investigate the nature of this unknown compound. The degradation kinetics of its aqueous solutions were examined. Ionspray mass spectrometry (ISP-MS) with flow-injection analysis (FIA) was applied [15,16]. Finally, an HPLC–MS method was used to gain more consistent information on this matter. Some hypotheses relating to the structure of the impurity were formulated.

2. Experimental

2.1. Samples

Amoxicillin sodium was from injectable pharmaceutical preparations obtained on the Italian market. Amoxicillin piperazine-2,5-dione, amoxicilloic acid and amoxicillin dimer and tetramer were synthesized according to the literature [14,17,18].

2.2. Chemicals

All the solvents were of HPLC grade. Acetonitrile was obtained from Merck (Darmstadt, Germany) and formic acid and potassium dihydrogen phosphate were obtained from Farmitalia Carlo Erba (Milan, Italy). Water was bidistilled. Polypropylene glycols PPG 1000 and PPG 2000 were from Aldrich (Milwaukee, WI). The mobile phases for HPLC were filtered through a 0.45 μm Nylon 66 membrane on a Millipore Milli-Q device. All other reagents were of analytical grade.

2.3. HPLC apparatus and chromatographic conditions

HPLC analyses were performed using a high pressure quaternary pump (HP 1050) equipped with an HP 1050 automsampler, an HP 1040M diode array detector and a MS-DOS 3D workstation, all from Hewlett-Packard (Avondale, PA, USA). The chromatographic column was a 10 μm LiChrosorb RP-18, 250 mm × 4.0 mm i.d. (Merck).

The mobile phases for linear gradient elution were prepared according to a reported method [19]: (A) potassium dihydrogen phosphate (pH 6; 0.01 M); (B) acetonitrile–potassium dihydrogen phosphate (pH 6; 0.01 M) (80:20 v/v). The linear gradient was from 5% to 70% of mobile phase (B) in (A) in 30 min at a flow rate of 1 ml min^{-1}.

All measurements were made at room temperature and the injection volume was 20 μl. The monitoring wavelength was 215 nm.

2.4. Micro HPLC–UV and micro HPLC–ISP-MS apparatuses and conditions

Mass spectral analysis was performed on a Perkin Elmer Sciex API 1 single-quadrupole instrument (Sciex, Thornhill, Ont., Canada). The mass spectrometer was equipped with an ISP interface set at a voltage of 5500 V and the nebulizer gas pressure (air) was 40 psi. Ultra high purity nitrogen (Rivoira, Chivasso, Turin, Italy) was used as the curtain gas in the Sciex API interface region at a constant flow rate of 0.6 l min^{-1}. Mass calibration was performed each day by continous infusion of a mixture of PPGs using a Harvard syringe pump (South Natick, MA) operating at a flow rate of 10 ml min^{-1}. Voltage parameters of the quadrupole offset and lenses used during sample analyses were: OR = 40, R0 = 30, R1 = 27, L9 = −249.

A Phoenix 20 dual syringe HPLC pump (Fisons Instruments, Milan, Italy) equipped with a Valco micro-injector (Valco, Houston, TX) and a 1 μl internal sample loop was used for the acquisition of both FIA–MS and HPLC–MS data.

For the FIA experiments the amoxicillin sodium solutions were injected into the mobile phase consisting of acetonitrile–water–formic acid (800:199:1, v/v/v) at a flow rate of 0.02 ml min^{-1}.

For the micro HPLC–UV experiments the pumping system was equipped with a 433 capillary detector with a 90 nl capillary flow cell (22

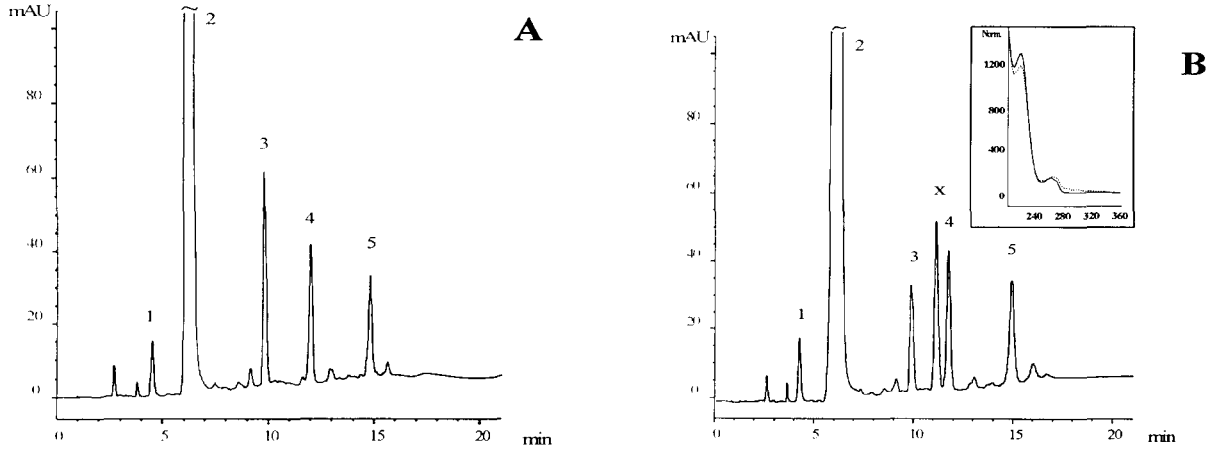

Fig. 1. Amoxicillin sodium HPLC chromatogram. (A) Sample injected 10 min after its preparation: (1) amoxicilloic acid; (2) amoxicillin; (3) piperazinedione; (4) dimer; (5) tetramer. (B) Sample injected immediately after its preparation: (1) amoxicilloic acid; (2) amoxicillin; (3) piperazinedione; (x) unknown impurity; (4) dimer; (5) tetramer. Inset: (—) zero order UV spectrum of amoxicillin; (...) zero order UV spectrum of the unknown impurity.

mm optical pathlength; Kontron Instruments, Munich, Germany) set at a monitoring wavelength of 215 nm.

The chromatographic column was a 5 μm Supelcosil LC-18-DB, 300 mm × 1 mm i.d. (Supelco, Bellefonte, PA). The mobile phase was water–acetonitrile–formic acid (250:49:1, v/v/v) at a flow rate of 0.02 ml min^{-1}. All measurements were carried out at room temperature.

The mass spectra collected in full-scan positive-ion mode were obtained by scanning over the range m/z 200–500 in 2 s.

2.5. Sample solutions

2.5.1. HPLC

For the purity determination a weighed amount of amoxicillin sodium was dissolved in mobile phase (A) at a concentration of 1 mg ml^{-1}. Amoxicilloic acid, amoxicillin piperazine-2,5-dione and amoxicillin dimer and tetramer were injected in mobile phase (A) at a concentration of 0.05 mg ml^{-1}. For the kinetic studies of the degradation of the unknown impurity, the following solutions of amoxicillin sodium were prepared: (a) 1 mg ml^{-1} in mobile phase (A) (pH 6); (b) 1 mg ml^{-1} in the solvent contained in the vial of the commercial drug package (glycine and sodium hydrate in water, pH 9.5). All the amoxicillin sodium solutions were injected immediately and at fixed time intervals after their preparation.

2.5.2. MS

Amoxicillin sodium was dissolved in mobile phase (A) at a concentration of 1 mg ml^{-1}. The solutions were analyzed immediately and at fixed time intervals after their preparation.

2.6. Quantitative analysis

The quantitative evaluation of the impurity content determined by HPLC was performed by the area percent method.

3. Results

Fig. 1A shows a chromatogram relating to the purity determination of a commercially available injectable preparation of amoxicillin sodium which is no longer obtainable. The injection of the sample solution was performed in mobile phase (A) (pH 6) within the time usually required for instrumental checking (about 10 min after sample preparation). The chromotographic profile provided evidence for the presence of some impuri-

ties. Amoxicilloic acid, amoxicillin piperazine-2, 5-dione and amoxicillin dimer and tetramer (retention times about 4.5, 10, 12 and 15 min respectively) were identified by comparison with reference compounds synthesized in this laboratory. The total amount of observed impurities was about 8%.

Fig. 1B shows a chromatogram relating to the above-mentioned sample solution but injected immediately after its preparation.

Comparison of the two chromatographic profiles gives evidence of the appearance of a new peak ("x", retention time 11 min) for the sample injected immediately after its preparation (Fig. 1B). It showed both zero order and derivative UV spectra similar to those presented by amoxicillin (Fig. 1B, inset) and was found in a significant amount (about 2%). The observed phenomenon was accompanied by a comparable decrease in the piperazinedione peak (3). The correlation between the unknown impurity and piperazinedione was demonstrated by injecting the sample solution immediately and at fixed time intervals after its preparation. The same phenomenon was observed at the mentioned injection times if amoxicillin sample was dissolved in the solvent contained in the vial of the commercial drug package (glycine and sodium hydrate in water, pH 9.5; Fig. 2). Experiments performed on some commercially available samples of different origin also provided evidence for the presence of the impurity, although to a lesser extent. In order to gain information concerning the structure of compound "x" and considering the difficulty in isolating the pure compound due to its high instability in solution, ionspray mass spectrometry with

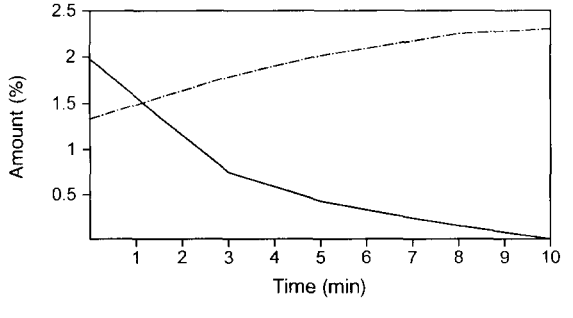

Fig. 2. Amounts of the unknown impurity (—) and piperazinedione (...) in amoxicillin sodium solutions analyzed by HPLC at fixed time intervals after their preparation.

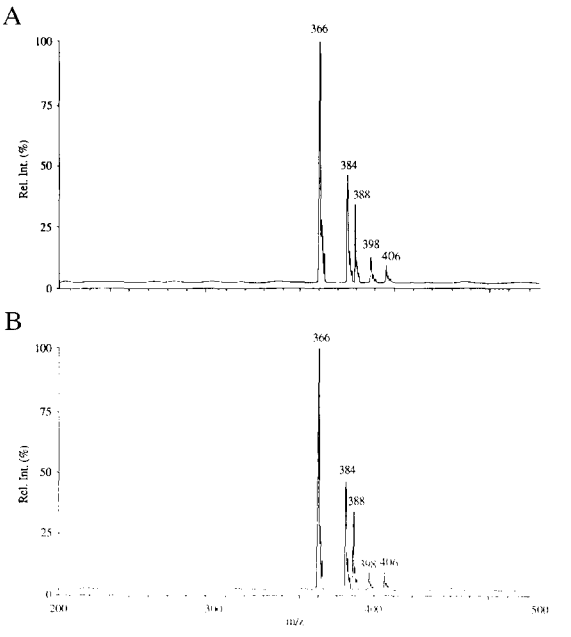

Fig. 3. FIA mass spectra of amoxicillin sodium. (A) Sample injected immediately after its preparation: amoxicillin and piperazinedione $[M + H]^+$ (m/z 366); amoxicilloic acid $[M + H]^+$ (m/z 384); amoxicillin and amoxicillin piperazinedione sodium adduct $[M + Na]^+$ (m/z 388); amoxicilloic acid sodium adduct $[M + Na]^+$ (m/z 406); $[M + H]^+$ (m/z 398): see Section 4. (B) Sample injected 10 min after its preparation: amoxicillin and piperazinedione $[M + H]^+$ (m/z 366); amoxicilloic acid $[M + H]^+$ (m/z 384); amoxicillin and amoxicillin piperazinedione sodium adduct $[M + Na]^+$ (m/z 388); amoxicilloyl methyl ester $[M + H]^+$ (m/z 398); amoxicilloic acid sodium adduct $[M + Na]^+$ (m/z 406).

FIA was utilized. This technique usually allows the observation of the molecular ions with little or no fragmentation [15]. Fig. 3A, which refers to the FIA–MS analysis of the amoxicillin sodium solution injected immediately after its preparation, shows two signals at m/z 366 and m/z 388 corresponding to the protonated amoxicillin molecule $[M + H]^+$ and to its sodium adduct $[M + Na]^+$ respectively. Signals at m/z 384 and m/z 406, which were assigned to the protonated amoxicilloic acid molecule $[M + H]^+$ and its sodium adduct $[M + Na]^+$ respectively, were also noticed. The signal relating to the piperazine-2,5-dione derivative could not be distinguished from the amoxicillin signal as the two products had the same m/z

value. Furthermore, the FIA–MS analysis exhibited a signal at m/z 398. To establish if this last signal could be related to compound "x" observed by HPLC–UV, the analysis was repeated on the amoxicillin solution at fixed time intervals after its preparation. The signal did not disappear after 10 minutes but its intensity decreased by about 13%, in disagreement with the degradation kinetics observed by HPLC (Fig. 3B). A more suitable technique (HPLC–MS) was then established for the identification of compound "x". Figs. 4 and 5 show the HPLC–UV and HPLC–MS chromatograms of the solutions injected at different time intervals ($t = 0$ and $t = 10$ respectively). The positive ISP mass spectra were taken from the

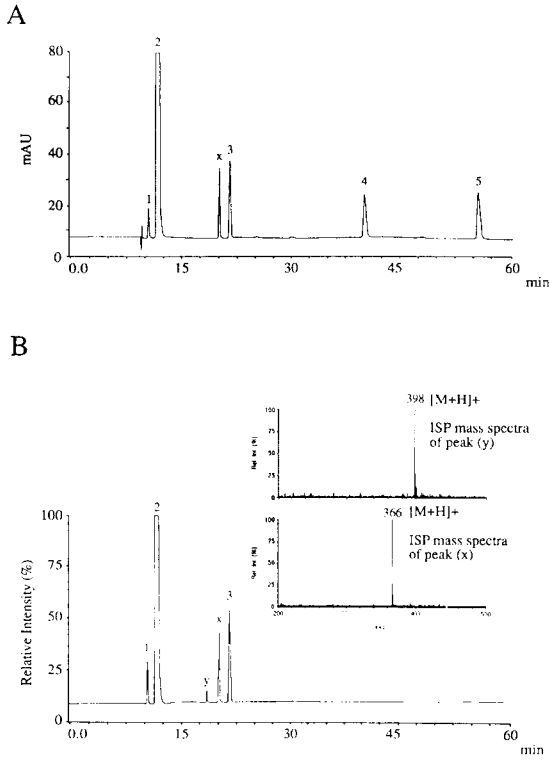

Fig. 4. Amoxicillin sodium solution injected immediately after its preparation. (A) Micro HPLC–UV chromatogram: (1) amoxicilloic acid; (2) amoxicillin; (x) unknown impurity; (3) piperazinedione; (4) dimer; (5) tetramer. (B) Micro HPLC–MS chromatogram: (1) amoxicilloic acid $[M + H]^+$ (m/z 384); (2) amoxicillin $[M + H]^+$ (m/z 366); (x) unknown impurity$[M + H]^+$ (m/z 366); (3) piperazinedione $[M + H]^+$ (m/z 366); (y) $[M + H]^+$ (m/z 398): see Section 4.

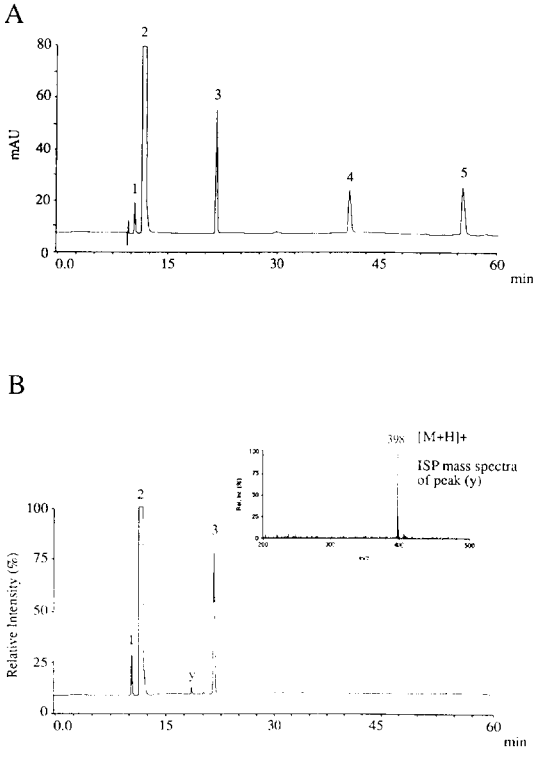

Fig. 5. Amoxicillin sodium solution injected 10 min after its preparation. (A) Micro HPLC–UV chromatogram: (1) amoxicilloic acid; (2) amoxicillin; (3) piperazinedione; (4) dimer; (5) tetramer. (B) Micro HPLC–MS chromatogram: (1) amoxicilloic acid $[M + H]^+$ (m/z 384); (2) amoxicillin $[M + H]^+$ (m/z 366); (y) amoxicilloyl methyl ester $[M + H]^+$ (m/z 398); (3) piperazinedione (m/z 366).

corresponding chromatographic peaks, allowing the identification of the investigated impurities by way of their molecular weights. The m/z 398 compound (y; retention time 18.7), detectable by HPLC–MS, was not observed in the HPLC–UV chromatogram at the corresponding retention time. Fig. 4 shows three compounds at m/z 366: amoxicillin (2; retention time 12.2), "x" (retention time 20.5) and amoxicillin piperazinedione (3; retention time 21.7). Compound "x" was not detectable in the HPLC–MS chromatogram of amoxicillin sodium injected 10 min after its preparation, according to the corresponding HPLC–UV chromatogram. As expected, this phenomenon was accompanied by a comparable increase in amoxicillin piperazinedione (Fig. 5).

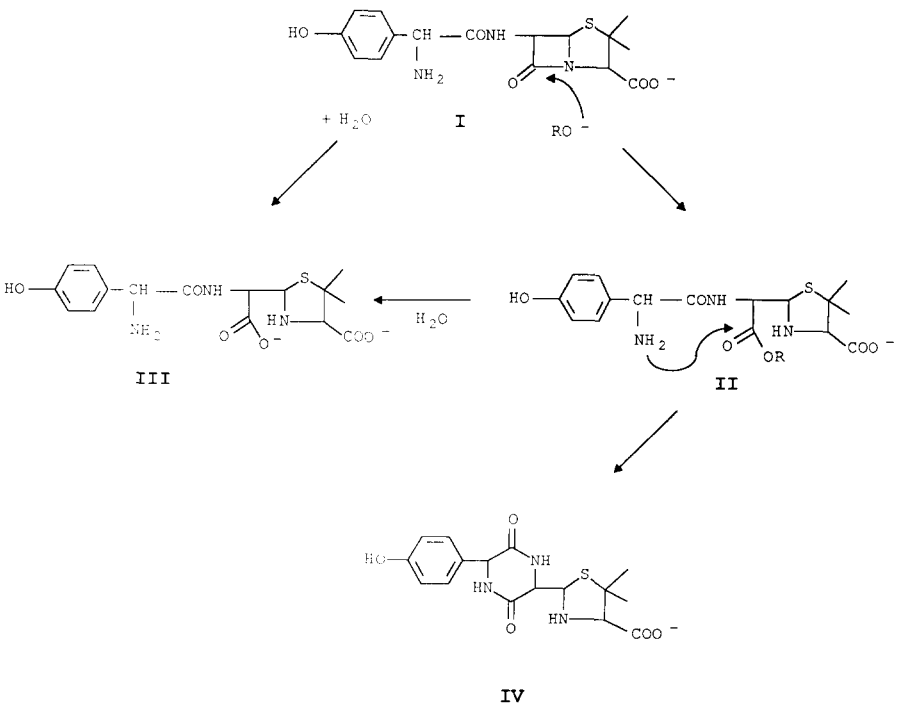

Fig. 6. Nucleophilic degradation mechanism of amoxicillin sodium [11]: (**I**) amoxicillin sodium; (**II**) amoxicilloyl ester; (**III**) amoxicilloic acid; (**IV**) piperazinedione.

4. Discussion

The experimental results obtained in the course of the HPLC analyses on the purity determination of amoxicillin sodium and the evidence of a correlation between piperazinedione and the unknown impurity led to some preliminary conclusions. It has been demonstrated that the degradation of ampicillin proceeds through a nucleophilic pathway with the initial formation of an α-aminobenzylpenicilloyl ester. It subsequently undergoes an intramolecular aminolysis at the side-chain amino group to produce a stable piperazine-2,5-dione derivative besides undergoing, to a much smaller extent, simple hydrolysis into the corresponding penicilloic acid [11]. Consequently, it would be reasonable to suppose that the degradation of amoxicillin, which differs from ampicillin only by the presence of a hydroxyl group in the acyl side-chain, can proceed through a similar nucleophilic pathway (Fig. 6). Therefore, the initial hypothesis was that the unknown compound could be an amoxicilloyl ester formed during the degradation pathway from amoxicillin to piperazinedione. However, this hypothesis was not supported by further experimental results. In fact, even though FIA–MS indicated the presence of m/z 398 signal that could be assigned to the protonated molecule $[M + H]^+$ of the amoxicilloyl methyl ester (Fig. 6; **II**: R = CH_3; $M_r = 397$), it could not be related to the "x" peak owing to the different degradation kinetics observed in solution for m/z 398 and "x". In addition, HPLC–MS analysis (Figs. 4 and 5) confirmed this finding, giving different retention times and molecular masses for the hypothesized methyl ester (y: retention time = 18.7; $M_r = 397$) and "x" (retention time = 20.5; $M_r = 365$). The amoxicilloyl ester was assumed to be present only in traces as it was not clearly identified in the HPLC–UV chromatogram.

Some tentative hypotheses on the chemical structure of the $M_r = 365$ labile impurity ("x") can be formulated considering that its molecular

mass and electronic absorption spectra correspond to those of amoxicillin and amoxicillin piperazinedione respectively:

"x" could be the enolic form of the amoxicillin piperazinedione; this assumption is however not supported by the fast interconversion rate of oxo-hydroxy tautomers;

"x" could be the (5*S*,6*R*) amoxicillin diasteroisomer; its lability in aqueous solution could be attributed to an easy hydrolytic opening of the (5*S*,6*R*) four-atom ring followed by epimerization at C-5 and concomitant transformation into amoxicillin piperazinedione (see Fig. 6). No supporting data however were found in the literature concerning the (5*S*,6*R*) amoxicillin diasteroisomer, which should arise exclusively from microbial syntheses, and the amoxicillin epimerization at C-5;

"x" could be the (5*S*,6*R*) amoxicillin piperazinedione diasteroisomer; its lability in aqueous solution could derive from an epimerization at C-5 [20].

The last hypothesis seems to be most likely. However, further studies are in progress in order to clarify definitively the mentioned assumptions.

5. Conclusions

A new impurity, unstable in aqueous solution, was found in some commercial injectable amoxicillin sodium samples. It was detected for a short time in the drug dissolved just in its own solvent vial (about 10 min). The described results give some indications on the structural nature of the new impurity (probably the amoxicillin piperazinedione (5*S*,6*R*) diasteroisomer). Further studies are in progress to give a definitive identification. The presence of the amoxicilloic acid methyl ester, an intermediate in the amoxicillin degradation process, was also hypothesized.

The toxicological and immunological significance of the described impurities should perhaps be evaluated, considering that the antigenicity of the penicilloic acid diasteroisomers and esters is not yet well established or defined. Furthermore, it should be mentioned that, according to the directions on the packaging of the injectable amoxicillin sodium preparation examined, the solution has to be injected "immediately" after its reconstitution. Consequently, at the injection time the drug could contain a considerable amount of the diasteroisomeric impurity.

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