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Hydrolysis of Penicillin G and Carbenicillin in Pure Water - As Studied by HPLC/ESI-MS

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Abstract : The hydrolysis of penicillin G, carbenicillin and ampicillin in pure water at room temperature was studied by high pressure liquid chromatography electrospray ionization mass spectrometry. Hydrolysis of ampicillin did not occur under these conditions; however, penicillin G and carbenicillin were completely hydrolyzed after seven days. A short interpretation of this difference is proposed. The mass spectrometric behaviour, namely ESI response and fragmentation pathway, of hydrolyzed penicillin G and hydrolyzed carbenicillin have been also discussed.

Keywords : penicillin, carbenicillin, hydrolysis, mass spectrometry, electrospray, liquid chromatography

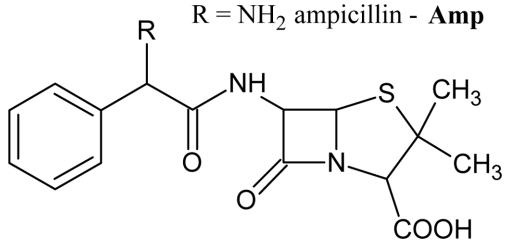
Introduction

Penicillin G (Pen) is the first antibiotic discovered by man still used to treat bacterial infections. Of course, a vast number of other penicillins are also widely used, e.g. aminopenicillin (ampicillin, Amp) and carboxypenicillin (carbenicillin, Carb) shown in Scheme 1.

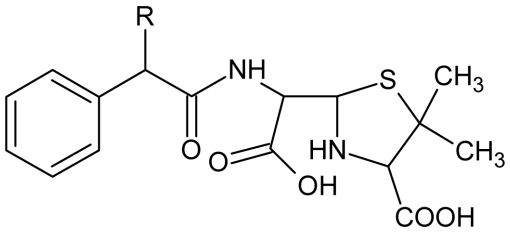
Like other drugs, penicillins can enter the environment which has stimulated huge amount of studies concerning their degradation under different conditions, e.g. photolysis degradation, sonochemical degradation, electrochemical degradation and others.[1-16] The first and most important, step of penicillins degradation is the hydrolysis of β-lactam ring, producing respective penicilloic acid (Scheme 2).[17]

In spite the fact that four-membered ring is strained and the activation energy of this reaction is low, at neutral pH and in the absence of other factors (e.g. heat, bacteria, metal cations) the hydrolysis reaction is slow.[18] However, we decided to evaluate the stability of penicillin G (Pen) and its conjugates (Amp, Carb) in pure water at room temperature. The question seems be so simple that it appears to be too trivial a matter of research, however in our opinion it is a fundamental question.

R = H penicillin - **Pen**
R = COOH carbenicillin - **Carb**
R = NH_2 ampicillin - **Amp**



Scheme 1. Structures of common penicillins.



Scheme 2. Structure of hydrolyzed penicillins (penicilloic acids).

Experimental

Penicillin G, ampicillin and carbenicillin (sodium salts) and pure penicillin G were obtained from Sigma-Aldrich (Poznań, Poland) and used without purification. It has to be

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added that each antibiotic contained a small amount of hydrolyzed compound (Pen_h, Amp_h, $Carb_h$). The water used was purified (deionized) by using Spring 20 demineralizer (Hydrolab, Straszyn, Poland). The conductivity of the purified water was < 0.1 μS/cm. The degradation tests were performed in 200 mL bottles filled with water and containing 5 mg of antibiotic (pH was about 6). The bottles were stored at room temperature (20-25°C), exposed to the day sunlight. Each day a 1 mL was collected from each bottle and put to the fridge. It should be stressed that for penicillins-containing water solutions stored in the fridge (4°C) we did not observe any hydrolysis of penicillins even after 30 days of storage (supplementary material). The samples were collected each day over 30 days and then subjected to HPLC/ESI-MS analysis. The collected samples were analysed one by one, and the HPLC/ESI-MS analysis were finished within 24 h (the ESI responses obtained at an interval of a few days may be different because of the fluctuations in pressure inside the mass spectrometer).

The HPLC/ESI-MS analyses were performed using a Waters model 2690 HPLC pump (Milford, MA, USA), a Waters/Micromass ZQ2000 mass spectrometer (single quadrupole type instrument equipped with electrospray ion source, Z-spray, Manchester, UK). The software used was MassLynx V3.5 (Manchester, UK). Using an autosampler, the sample solutions were injected onto the LiChrospher C18 column (5 μm, 125 × 4 mm i.d., Phenomenex). The injection volume was 5 μL. The solutions were analysed by using linear gradient of CH_3CN-H_2O with a flow rate of 0.4 mL/min. The gradient started from 0% CH_3CN - 95% H_2O with 5% of a 10% solution of formic acid in water, reaching 95% CH_3CN after 10 min, and the latter concentration was maintained for 7 min.

The ESI mass spectra were recorded in the *m/z* range 100-1000, in positive and negative modes simultaneously (during the HPLC/ESI-MS analyses the mass spectrometer was switched in the fast mode between the positive and negative ion modes) The electrospray source potentials were: capillary 3 kV, lens 0.5 kV, extractor 4 V and cone voltage 20 V (unless indicated otherwise). Cone voltage has the most profound effect on the mass spectra obtained. Increase in this parameter leads to the so-called "in-source" fragmentation/dissociation but a too low cone voltage may cause a decrease in sensitivity. The source temperature was 120°C and the desolvation temperature 300°C. Nitrogen was used as the nebulizing and desolvation gas at the flow rates of 100 and 300 L/h, respectively.

Results and Discussion

At first, it should be emphasized that hydrolysis of ampicillin occurred very slowly in pure water at room temperature.[19,20] During the degradation test a small amount of ampicillin penicilloic acid was formed. However, the amount of ampicillin did not decrease to more than 90% of initial amount. Therefore, the ESI-MS data obtained for ampicillin are not shown here.

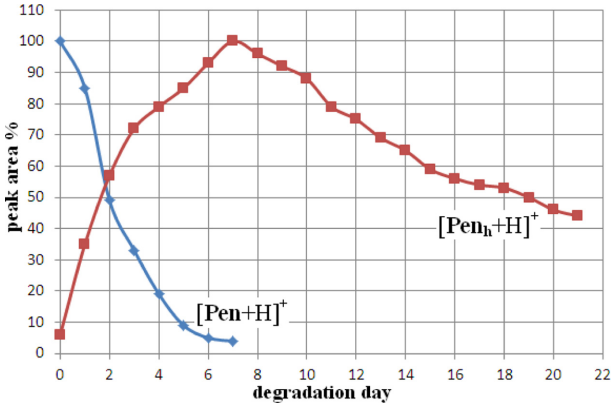


Figure 1. The breakdown plots of chromatographic peak areas of $[M+H]^+$ ions against days of degradation test obtained for penicillin G (sodium salt).

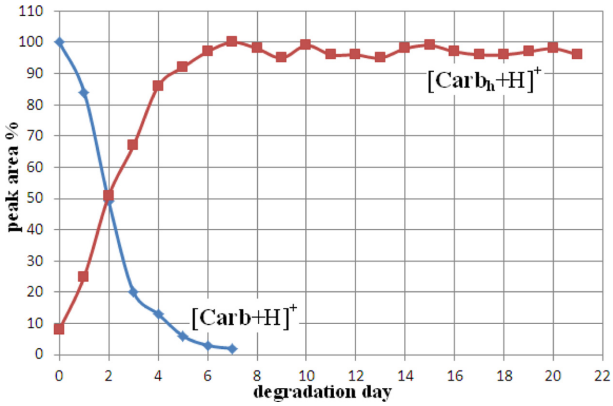
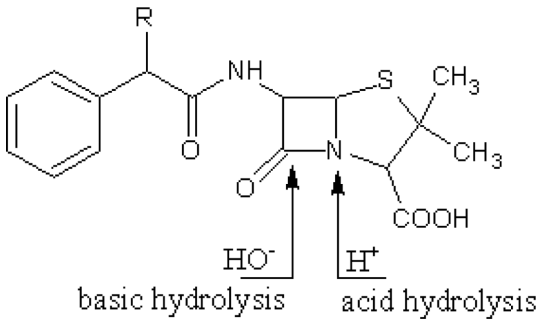


Figure 2. The breakdown plots of chromatographic peak areas of $[M+H]^+$ ions against days of degradation test obtained for carbenicillin (sodium salt).

Hydrolysis of penicillin G and carbenicillin occurred relatively fast. Figure 1 and 2 show the breakdown plots of chromatographic peak areas of $[M+H]^+$ ions (M stands for neutral antibiotic molecule) against days of degradation test (for clarity it is shown for 20 days). The peak areas obtained in arbitrary units were converted for percentages (the largest peak is 100%). As clearly results from Figure 1 and 2, after seven days the penicillin G and carbenicillin (sodium salts) were completely hydrolyzed.

We have also performed the experiment for pure penicillin G (not sodium salt). As shown in supplementary material, the results are very similar to that obtained for penicillin G sodium salt.

In other words penicillin G and carbenicillin stored in water at room temperature after one week lose their antibiotic activities. It is a matter of discussion if this period is short enough or special efforts should be taken to



Scheme 3. The beginning of basic and acid hydrolysis of penicillins.

accelerate degradation of these antibiotics.

Hydrolysis of penicillin G or carbenicillin is very similar, however, the stabilities of hydrolyzed antibiotics (Pen_h and $Carb_h$) are different (Figure 1 and 2). Namely, hydrolyzed carbenicillin is stable under the conditions used, whereas hydrolyzed penicillin slowly undergoes degradation process. We have looked for its degradation products, however the expected signals, e.g. ions at *m/z* 309 or 235 were not detected,[9] and we have not detected any others signals of degradation products.

The correlation between penicillins structures and the rate of their hydrolysis under basic conditions has been a matter of discussion.[21] The key question is why ampicillin is stable under the conditions used in this work, whereas penicillin G and carbenicillin undergo hydrolysis. Abiotic (non-enzymatic) hydrolysis can occur due to the nucleophilic attack on carbonyl carbon atom of β-lactam ring (basic hydrolysis) or protonation of nitrogen atom of β-lactam ring (acid hydrolysis), as shown in Scheme 3.[17]

If under the neutral conditions we would deal with basic mechanism, the high stability of ampicillin in comparison to those of penicillin G and carbenicillin could not be expected. It is reasonable to suppose that we deal with acid mechanism. Ampicillin is protonated at amino group since this is the most basic site.[22] Thus protonation at nitrogen atom of β-lactam ring does not occur for ampicillin as a consequence hydrolysis does not occur. However, protonation at nitrogen atom of β-lactam ring for penicillin G and carbenicillin is possible, so as a consequence their hydrolysis takes place.

HPLC/ESI-MS analysis of penicillins in the negative ion mode is much less common than their HPLC/ESI-MS analysis in the positive ion mode, since under ESI(+) conditions we deal with a higher response than under ESI(-) conditions. However, we have performed the HPLC/ES-MS analysis in both positive and negative ion mode. As clearly results from the chromatograms shown in Figures 3-6 (second degradation day), the ESI(+) response of hydrolyzed penicillin G and hydrolyzed carbenicillin is much higher than their ESI(-) responses (although, penicilloic acid has been successfully analyzed in negative ion mode as well[23]).

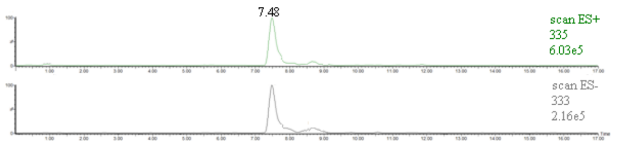


Figure 3. Exemplary single ion chromatograms obtained for penicillin G. $[M+H]^+$ *m/z* 335, $[M-H]^-$ *m/z* 333. It is evident that ESI(+) response of penicillin G (maximum abundance is $6.03x10^5$) is much higher than its ESI(-) response (maximum abundance is 2.16×10^5).

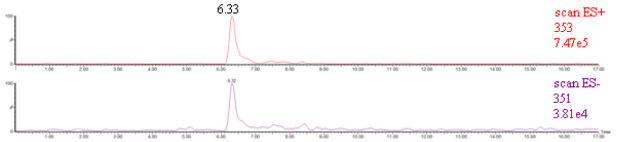


Figure 4. Exemplary single ion chromatograms obtained for hydrolyzed penicillin G. $[M+H]^+$ *m/z* 353, $[M-H]^-$ *m/z* 351. It is evident that ESI(+) response of hydrolyzed penicillin G (maximum abundance is 7.47×10^5) is much higher than its ESI(-) response (maximum abundance is 3.81×10^4).

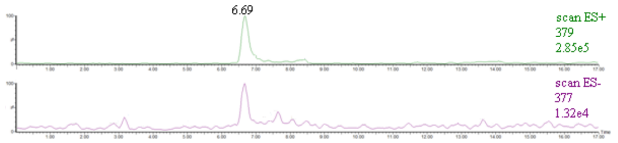


Figure 5. Exemplary single ion chromatograms obtained for carbenicillin. $[M+H]^+$ *m/z* 379, $[M-H]^-$ *m/z* 377. It is evident that ESI(+) response of carbenicillin (maximum abundance is 2.85×10^5) is much higher than its ESI(-) response (maximum abundance is 1.32×10^4).

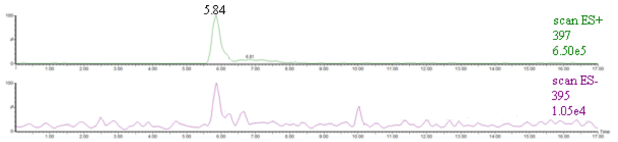


Figure 6. Exemplary single ion chromatograms obtained for hydrolyzed carbenicillin. $[M+H]^+$ *m/z* 397, $[M-H]^-$ *m/z* 395. It is evident that ESI(+) response of hydrolyzed carbenicillin (maximum abundance is 6.50×10^5) is much higher than its ESI(-) response (maximum abundance is 1.05×10^4).

The high ES(+) responses of hydrolyzed penicillin G and hydrolyzed carbenicilin are most probably related with the high proton affinity (gas basicity) of thiazolidine ring.[22] Thus, from the analytical point of view, it is worth knowing the fragmentation pathways of hydrolyzed penicillin G and hydrolyzed carbenicillin, which occur under the ESI conditions in the positive ion mode.

The fragmentation pathways of penicillin G and

carbenicillin have been already reported, including interpretation of the product ions.[22] Fragmentation pathway of hydrolyzed penicillin G has been reported, however without interpretation of its product ions.[13,24] The fragmentation pathway of hydrolyzed carbenicillin, to the best our knowledge, has not been reported yet. We have found that fragmentation of ions $[Pen_h+H]^+$ and $[Carb_h+H]^+$ is identical and corresponds mainly to the decarboxylation process and/or formation of thiazolidine ring-containing product ions. In the supplementary material the respective ESI(+) mass spectra are shown, including interpretation of the most abundant product ions. It is worth mentioning that the fragmentation of ion $[Pen_h+H]^+$ has been found to be different from that reported by Li et al., namely, the product ion at *m/z* 194 has not been detected.[25] It is also worth adding that fragmentation pathways of a number of penicilloic acids (including hydrolyzed carbenicillin) under the FAB conditions have been described in details, in both positive and negative ion mode.[26] However, nowadays, FAB has no analytical importance.

Although the purpose of this work was to check if degradation of penicillin G and carbenicillin can occur in pure water, we have also performed the degradation test in river water (the Warta river, Poznań, Poland). As shown in the supplementary material, the degradation of both antibiotics occurred in the river water (environmental-like conditions) in a similar manner as in pure water.

Conclusions

After seven days, the penicillin G and carbenicillin were completely hydrolyzed in pure water at room temperature, thus they lost the antibiotic activities. It is reasonable that the hydrolysis occurred due to the protonation of nitrogen atom of β-lactam ring (acid mechanism). In spite the fact the hydrolysis products contain an additional carboxylic group, their ESI(-) responses are very low in comparison to the ESI(+) responses. Fragmentation pathways of protonated hydrolyzed penicillin G and protonated hydrolyzed carbenicillin correspond mainly to the decarboxylation process and/or formation of thiazolidine ring-containing product ions.

Aknowledgement

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Supporting Information

Supplementary Information is available at https://drive.google.com/open?id=1YMUAam75PNKDcCQoMXdj_BffGREDMuLX

†Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: https://drive.google.com/open?id=1YMUAam75PNKDcCQoMXdj_BffGREDMuLX

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