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Detection of amoxicillin-diketopiperazine-2', 5' in wastewater samples

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The short half-life of aminopenicillin antibiotics in the aquatic environment put to the challenge the detection of their degradation products among environmental hydro-chemists. In a quest to study the occurrence of a new emerging micro-pollutant in the aquatic environment we attempted this by analyzing samples from a wastewater treatment plant for a major degradation product of amoxicillin (i.e., amoxicillin-diketopiperazine-2', 5') using a high-performance liquid chromatography technique coupled with tandem mass spectrometry method. ADP was repeatedly detected in all wastewater and effluent samples (18) from which it was extracted. To the best of our knowledge, this is the first study that evidently proves the occurrence of the chemically stable form of AMX, its Diketopiperazine-2', 5', in wastewater and effluent samples. Furthermore, penicillins are known to cause most allergic drug reactions. There is a risk that residues of hypersensitivity-inducing drugs, such as penicillins and their degradation products, may elicit allergic reactions in human consumers of water and food of animal origin.

Keywords: Aminopenicillins, amoxicillin-diketopiperazine-2', 5', degradation product, wastewater, WWTPs.

Introduction

Penicillins make up one of four groups that belong to the β -Lactam class of antibiotics. Within this group, the most consumed subgroup is the aminopenicillins, which include ampicillin, amoxicillin, epicillin and bacampicillin. These semi-synthetic penicillin-like antibiotics are distinct from the “conventional” penicillins by the addition of an extra amine group in their side chain. They are used in the treatment of a variety of infections such as upper respiratory tract, urinary tract, meningitis and *Salmonella* infections, and effective for both Gram-positive and Gram-negative bacteria. Therefore they are labeled as “broad-spectrum penicillins”.^[1] There have been a few attempts to detect traces of penicillins in general and aminopenicillins in particular, especially amoxicillin (AMX), in environmental samples^[2–10] and hospital sewage water.^[11,12] However, the outcome of detecting their traces was always going to be difficult to achieve. One reason for that is the incompatibility of most analytical methods to extract penicillins. Several of the above methods were developed to extract var-

ious classes of antimicrobials (or other pharmaceutical substances) simultaneously but were not aimed specifically for penicillins. Moreover, recently developed methods for the extraction of simply penicillins did not show better results in detection of these compounds via high performance liquid chromatography with UV-diode array detection (HPLC–UV-DAD)^[13], or even coupled with electrospray ionization tandem mass spectrometry (ESI-MS/MS).^[14,15] Another reason for the inability to detect penicillins in environmental samples is the unique chemical structure of these antibiotics, which are readily degraded in both acidic and basic media, due to the opening of their strained β -Lactam ring by β -Lactamase, a widespread enzyme in bacteria, or by chemical hydrolysis.^[1]

Oppositely some traces of AMX were found in 3 out of 8 wastewater treatment plants (WWTPs) in an analytical campaign in Italy.^[16] It was detected at a concentration of 120 ng/L, 15 ng/L and 25 ng/L in Palermo, Latina and Varese-Olona WWTPs, respectively. In the UK, it was detected in 3 out of 4 sampling locations: 39–49 ng/L in Merthyr Tydfil, 198–245 ng/L in Trefforest Estate and 56–60 ng/L in Cardiff but could not be detected in 2 sampling locations in the river Warta in Poland.^[17] Moreover, it was found at a maximum concentration of 280 ng/L in the raw sewage and a maximum concentration of 30 ng/L in the effluent of a conventional treatment plant in Brisbane, Australia.^[18]

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Worldwide, there seems to be a misconception among environmental hydro-chemists concerning the occurrence of aminopenicillins in the aquatic environment. On the one hand, they are the most consumed antibiotics worldwide, and therefore are expected to be found at a relative high concentration in wastewater and surface water. Yet on the other hand, these compounds are tremendously difficult to trace. Given that the β -Lactam ring is susceptible to open, it is likely that the parent compound undergoes a degradation process. If that is the case, it is almost impossible to trace them and effort should be placed on determining their degradation products in the environment, a task that was never challenged before. Therefore, we challenged this task by attempting to detect traces of a major degradation product of AMX, its Diketopiperazine-2', 5' form (ADP) by analyzing wastewater samples (i.e., raw sewage and effluent).

Materials and methods

Chemicals

Amoxicillin trihydrate (AMX) (99.8%) analytical standard was purchased from Sigma Aldrich (Rehovot, Israel). Acetonitrile (AcN), methanol (MeOH) and water (all ultra pure LC/MS grade) were provided from Biolab (Jerusalem, Israel). Trifluoroacetic acid (TFA) (extra pure 99%) was purchased from Acros Organics (Yehud, Israel) and ortho-phosphoric acid (H_3PO_4) (HPLC grade 85%) from Fluka (Rehovot, Israel). Sodium chloride (NaCl) (analytical grade 99.5%) was provided from Biolab and sodium phosphate dibasic (Na_2HPO_4) (analytical grade 98%–100.5%) from Sigma Aldrich.

Sampling procedure

Both the entrance (i.e., raw sewage) and exit (i.e. effluent) of a WWTP in the Sharon Region, Central Israel were sampled several times, over a period of ten weeks, from February 27, 2008 to May 14, 2008. Each sample was collected in duplicates of 4L amber glass bottles, for the prevention of potential photo-degradation. The bottles were initially rinsed with the specific type of water (e.g., raw sewage or effluent) prior to filling, preserved in a plastic cooler packed with ice, and transported with no delay to the laboratory for storage in darkness at 4°C and for further pretreatment and sample preparation. Prior to the preparation stage, samples were measured for their pH (~ 7.5) using a Mettler Toledo MA235 pH meter.

Filtration

Wastewater samples were filtered through four different pore size filters to remove suspended matter: GF/D (2.7

μm), GF/A (1.6 μm), GF/F (0.7 μm) Glassfiber discs (Whatman®) and 0.45 μm PVDF Stericup® vacuum-driven filtration and storage device (Millipore). In the filtration of effluent, only a Stericup device was used.

Solid-phase extraction (SPE)

Early experiments at our laboratory demonstrated that ADP was barely retained on C-18 and phenyl reverse-phase sorbents, and on hydrophilic-lipophilic balanced cartridges with a copolymer sorbent (HLB). Consequently, Oasis® MAX (Waters), a Mixed-mode Anion-eXchange and reversed-phase sorbent was found as optimal. The cartridges were conditioned prior to sample loading with 5 ml MeOH, 5 ml H_2O and 5 ml phosphate buffer (pH 7.5). When the sample loading step was completed, the cartridges were washed with 2 ml solution comprised of AcN: H_2O (3:97) also at pH 7.5, to remove undesired organic interferences, and in the last step analytes were eluted with 2 ml of 1M NaCl solution comprised of AcN: H_2O (15:85) at pH 4. For an effective recovery of the analyte, the samples were evaporated in a temperature of 45°C using a gentle stream of nitrogen gas and were re-dissolved with H_2O .

Liquid chromatography

The LC system was a HP 1100 Agilent comprised of a refrigerated autosampler, binary pump, online vacuum degasser, UV-DAD and large column compartment. The system was controlled through multi-technique HP ChemStation software (version 10.1). The mobile phase was comprised from two different solvents, where channel A was MeOH and channel B was H_2O with 0.03% TFA. The chosen stationary phase was a RP-phenyl column from ACE® (250 mm in length, 2.1 mm I.D, 5 μm particle size and 100 Å pore size). The column was assisted by a matching pre-column (13 mm; 2.1 mm I.D) packed with the same stationary phase, sitting in a metallic pre-column guard. The injection volume was set at 100 μL , the flow rate at 0.5 mL/min, the column temperature at 28°C, and the UV spectrum was set to acquire data in the molecules' typical absorbance at 3 selected bands (220, 230 and 275 nm). The program's optimal gradient mobile phase composition is presented in Table 1.

Table 1. HPLC optimal gradient program.

Time (min)	A (%)	B (%)
0	5	95
15	75	25
17	75	25
19	5	95

Sample Enrichment

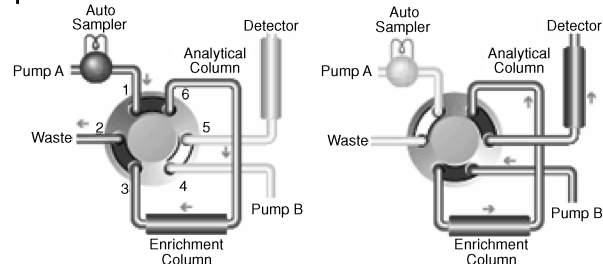


Fig. 1. Typical two-position valve.

Mass spectrometry

A Finnigan LCQTM MS was used to detect the analyte. It was tuned with direct infusion of 1 $\mu\text{g}/\text{ml}$ solution via syringe pump in the positive ion mode and with flow rate of 80 $\mu\text{L}/\text{min}$. MS data acquisition was achieved with the LCQ Tune Plus window. The apparatus was tuned to detect in the MS/MS mode, where consequently its sensitivity was enhanced. An ion in the center of the mass range (precursor ion) was selected to optimize the parameters in the single reaction monitoring (SRM) scan type, including optimization of the collision energy (CE) in the ion trap. The MS/MS scan parameters were as follow: the precursor ion $[\text{M}+\text{H}]^+$ was $m/z = 366$ (isolation width = 3 m/z). The normalized CE was 20%. The product ion of the highest intensity for SRM was $m/z = 160$, (isolation width = 1 m/z). The tune file included the following parameters: spray needle voltage = 4.5 kV, capillary voltage = 13 V, tube lens offset = 15 V, sheath gas flow-rate = 95 (arbitrary units), auxiliary gas flow-rate = 48 (arbitrary units) and capillary temperature 200°C. Instrument control, data acquisition and evaluation were performed with Xcalibur software.

Production of ADP

ADP was produced in our laboratory as a standard (after degradation process of AMX) and identified with multiple techniques. A HP 1050 HPLC semi-preparative system was used with a RP-C18-Vydac column (250 mm in length, 10 mm I.D., 10 μm particle size). ADP was collected using an online sample enrichment pre-column packed with C-18. The sample enrichment process was made with a two-position valve (Fig. 1) using a standard solution of AMX

(100 $\mu\text{g}/\text{mL}$) after a degradation process. In the first stage, the sample was drawn with pump A, where consequently ADP was retained on the pre-column and the rest of the solution was passed to the waste collector. In the second stage, using pump B (HPLC pump), it was eluted from the pre-column into the C-18 column with a binary gradient of two different solvents: MeOH and H₂O with 0.03% TFA. ADP was collected in a glass tube, lyophilized to dryness and was then re-dissolved in water once again.

Results and discussion

The scientific literature indicates that there have been few attempts to manipulate chemically AMX into its major degradation products in laboratory experiments.^[19–22] This manipulation was either by derivatisation or by breaking down the molecule and analyzing its degradation products using HPLC-MS/MS, exclusively for the pharmaceutical industry (e.g., drug purity) or medical research purposes. In mid eighties, researchers^[23] isolated and examined the two main isomers of AMX-diketopiperazine-2', 5' (2R and 2S epimers) using HPLC-UV, nuclear magnetic resonance (NMR) and MS techniques. The suggested degradation pathway of AMX in an aqueous medium (Fig. 2) starts with the opening of the four-membered β -Lactam ring by hydrolysis and yields the intermediate AMX-penicilloic acid, which contains an extra free carboxylic acid group. Subsequently, this intermediate rapidly forms a more stable six-membered ring product, the AMX-Diketopiperazine-2', 5'.^[22]

In contrast to the mentioned above, ADP was never extracted and detected from any environmental aquatic matrices. Therefore, in this study, the identification of the two isomers of ADP (A and B) prior to wastewater samples were made with various analytical methods. Figure 3 shows a HPLC chromatogram with the identification of AMX and the two isomers of ADP (A & B). Additionally, Figure 4 shows a comparison between the three UV spectra, which represents the absorbance of the isomers in relation to AMX, showing that both isomers have similar spectra to their parent compound (AMX).

Furthermore, ADP was identified using MS full scan method and the results were then compared with the findings in the literature.^[22,23] The top chromatogram in Figure 5 shows the detection of the two isomers of ADP

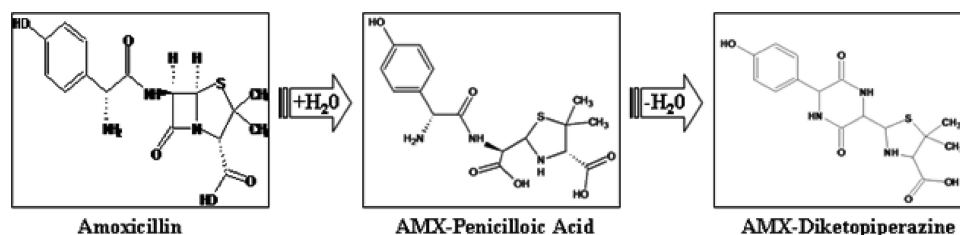


Fig. 2. Suggested degradation pathway of Amoxicillin in an aqueous medium.

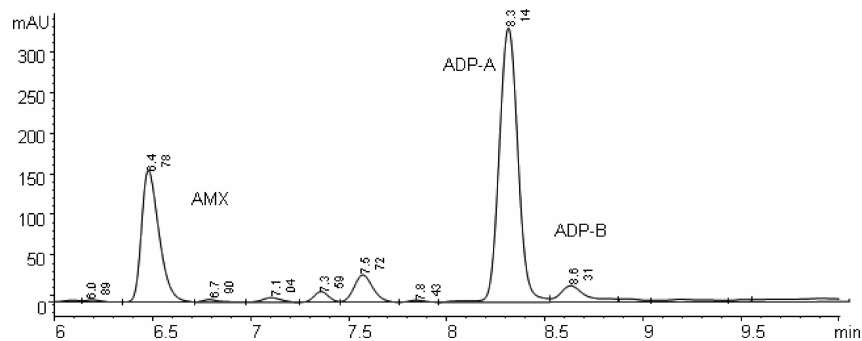


Fig. 3. HPLC chromatogram showing the identification of AMX and two isomers of ADP.

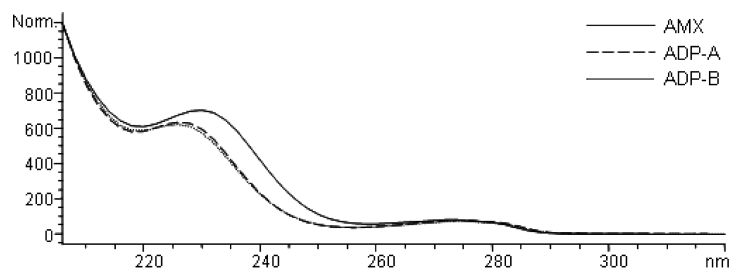


Fig. 4. Comparison between the UV spectra of the two isomers of ADP and AMX.

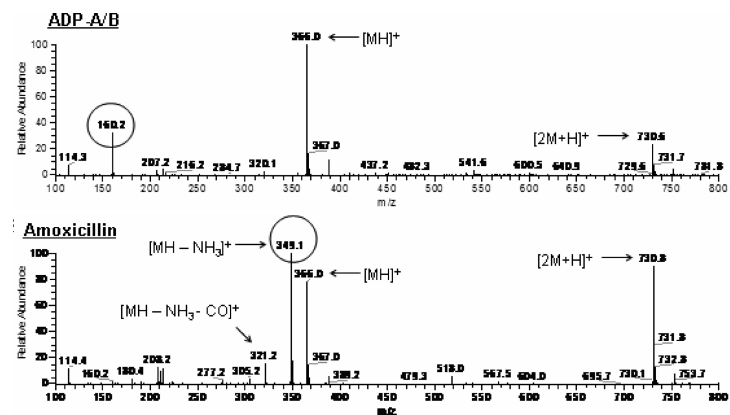


Fig. 5. MS chromatogram showing the detection of ADP-A, ADP-B and AMX.

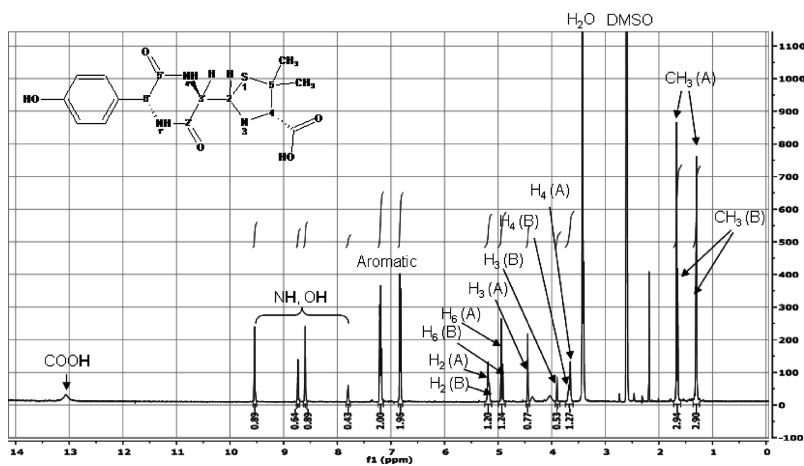


Fig. 6. NMR spectrum showing the identification of ADP.

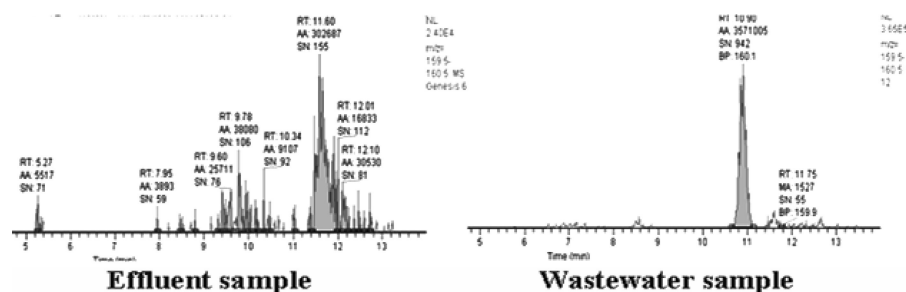


Fig. 7. A representative (March 23, 2008) MS/MS chromatogram showing the detection of ADP in wastewater and effluent.

at $[M+H]^+ = 366$ m/z with typical fragments at $m/z = 160$, corresponding to $[C_6H_9NO_2S+H]^+$, which is a typical fragment for all the penicillins. The bottom chromatogram indicates the detection of AMX at $[M+H]^+ = 366$ m/z as well; only this time, the main fragment is at $m/z = 349$, corresponding to the loss of NH_3 , which is typical for AMX.

The final identification of ADP in the current study was made with NMR, and in this case as well, the results were compared with previous findings in the literature.^[23] Figure 6 presents the identification and the structure elucidation of both ADP isomers (A, B) using the NMR spectrum.

The unambiguous identification of ADP enabled us to detect its traces in wastewater samples. Therefore, Figure 7 shows a representative (March 23, 2008) MS/MS chromatogram of effluent and wastewater samples (not spiked). It is important to emphasize that ADP was repeatedly detected in all wastewater and effluent samples (18) which it was extracted from.

The actual meaning of the incapability to detect penicillins in general, and AMX in particular, in wastewater and effluent samples is that either they do not exist at all, due to hydrolysis degradation processes, or that their concentration in this media is lower than the reported limits of detection in the relevant literature. To the best of our knowledge, this is the first study that evidently proves the occurrence of the chemically stable form of AMX, Diketopiperazine-2', 5', in wastewater and effluent samples, even though it was impossible to quantify these findings in the current study.

Moreover, penicillins are known to cause most allergic drug reactions. It is estimated that the overall prevalence of allergy to penicillin in human population is 3–10%. There is a risk that residues of hypersensitivity-inducing drugs, such as penicillins, may elicit allergic reactions in human consumers of water and food of animal origin.^[24]

Allergic reactions to water/food containing residues of penicillin are generally restricted to dermatological reactions such as urticaria, skin rash, polymorphic exanthems, asthma, and hypertension.^[25] However, there are reports of life-threatening anaphylactic shock reactions in (pre)sensitized subject after consumption of food (meat and milk) containing penicillin residues.^[24–31]

As mentioned before, the diketopiperazine-2', 5' is a possible product of the AMX-penicilloic acid (Fig. 2), which

is definitely occur in food^[31] and according the results of this paper, in water sample as well (diketopiperazine-2', 5'). De Baere et al.^[32] described a prolonged presence of the AMX-penicilloic acid in food samples, consequently, the occurrence of diketopiperazine-2', 5' as a penicillin drug degradation product, in food and water consumed by human, has a potential to cause an allergic drug reactions. Thus, there is an enormous importance to trace the presence of diketopiperazine-2', 5' in the environment and especially in various water resources and food.

Additionally, because the occurrence and fate of aminopenicillins in the aquatic and terrestrial environments cannot be ruled out, future studies should continuously measure the concentrations of these antibiotics, and especially of their degradation products, some of which are currently being identified via HPLC-MS/MS and NMR in an on going research at our laboratory.

Acknowledgments

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