# Investigation of dipyrone metabolites by applying municipal wastewater treatment technologies

PhD thesis

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# I Introduction

One of the most significant environmental problems of our time is the discharge of pharmaceutical residues into wastewater, natural waters and even in drinking water. Not only the amount of consumed medicines is growing gradually but also in several cases, there is no suitable microbial biochemical mechanism available at wastewater treatment plants to degrade these substances to bacteria populations. Nowadays, extensive researches are conducted worldwide to monitor xenobiotics excreted by the human body, since these metabolites are not only occurring in large quantities in the environment but they are converted during their biotransformation into compounds that persist in the environment risking, posing a threat, among others, to the purity of water resources. Up to now, less attention has been paid to pharmaceutical residues, household chemicals and even clinical diagnostic substances but nowadays, they are in the limelight. These compounds and their biologically active/inactive metabolites are primarily released into surface waters through untreated or treated wastewater discharges. Pollution of natural waters is particularly important in order to maintain the ecological equilibrium of the water compartments, since the life cycle of (micro)organisms living

in water is altered by contaminants and they become vulnerable. Undetectable or unobservable effects are particularly alarming for these microorganisms, because they may be synergistic. Thus, the major changes become unobservable, while the synergistic effects trigger an irreversible process in the natural adaptation and counter-selection.

## II Objectives

### 1. Determination of dipyrone metabolites in the wastewater of two wastewater treatment plants in Budapest and one in the suburban area by HPLC-MS

In Hungary, dipyrone is one of the analgesics sold in the highest quantity. The occurrence of dipyrone metabolites in untreated and treated communal wastewater has not yet been investigated in Hungary. Therefore, our objective was to develop a HPLC-MS method suitable for their quantitative determination in low concentration and enabling to monitor the fate of the metabolites of this medicine from wastewater to surface waters. The main metabolites of this active substance excreted from the human body are the following: 4-aminoantipyrine (4-AA), 4-acetylaminoantipyrine (4-AAA), 4-formylaminoantipyrine (4-FAA) and 4-methylaminoantipyrine. Little is known about the impact of

these compounds on the environment but acute toxicity has been detected by organisms that live in water for all these metabolites. Therefore, their study in water environment is extremely important.

## 2. Comparison of different wastewater treatment technologies

In possession of the abovementioned method, the aim was to study the efficiency of two different wastewater treatment technologies on the removal of the target molecules. For this purpose, the North- and South-Pest Wastewater Treatment Plants using activated sludge technology and Organica Waters Zrt. in Telki that operates a so-called fixed biofilm reactor system were chosen. In this latter plant, wastewater treatment is performed by microorganisms living in the biofilm formed on the roots of aquatic plants and/or on a synthetic carrier.

## 3. Monitoring of wastewater treatment plants

Our other goal was to monitor the metabolites of dipyrone in the chosen wastewater treatment plants. We aimed to investigate whether the seasonal change had an impact on the concentration of the selected pharmaceutical metabolites in the untreated wastewater after preliminary sedimentation and in the biologically treated water, as well, investigating, at the

same time, the possible seasonal patterns of the removal efficiency for dipyrone metabolites. A further task was to follow the daily changes in concentration of dipyrone metabolites. Another objective was to study the influence of chlorination applied after the biological wastewater treatment aiming disinfection on the concentration of dipyrone metabolites discharged in the Danube River, close to the Ráckeve resort area.

## III Materials and methods

### 1. Sample collection

For the first part of the investigation, samples were collected from the settled (influent) and biologically treated (effluent) waters of the selected communal wastewater treatment plants of North- and South-Pest in February 2011. Samples were also collected from the untreated and treated wastewater of the settlement of Telki located near Budapest in December 2010. For the second part of the study, samples were collected monthly from the influent and effluent water of the same wastewater treatment plants between July 2011 and March 2012, continuing the previously started research. Additionally, samples were collected from the biologically treated water of the South-Pest Wastewater Treatment Plant

from July 2011 until September 2011 before and after chlorination. The *intra*day variation of dipyrone metabolites was studied by sampling once for 24 hours with a frequency of 6 hours at South-Pest Wastewater Treatment Plant in May 2011.

## 2. Sample preparation and storage methods

After filtration of the samples through a GF/A Ø 125 mm glass microfiber paper, each 500 mL sample was subjected to solid phase extraction (SPE) and loaded onto Phenomenex Strata X 33μ 200 mg/3 mL reversed-phased cartridges aiming at separation from matrix and pre-concentration. The SPE columns were conditioned twice with 3 mL methanol, then with 3 mL of pH=8 ammonium acetate solution. After the loading of the 500 mL sample, the cartridges were washed twice with 3 mL of a 5 V/V% methanol (pH=8 with acetic acid) solution and, then, elution was performed with 3 mL methanol containing formic acid in 0.1 V/V% added twice The methanolic SPE eluates were stored at 4 °C for maximum two weeks prior to further analysis.

In the first part of the investigation, the samples needed to be further concentrated, therefore each 1 mL of the eluting solution was pre-concentrated to 50 μL in a rotary evaporator. Then, the volume of each sample was adjusted to 100 μL with

deionized water and filtered through a 0.22 μm pore size Nalgene filter. The resulted solutions were injected into the HPLC-MS system.

**3. Preparation and purification of standards**

Three compounds were synthesized at the Institute of Biochemistry and Medical Chemistry at the University of Pécs, while the commercially available 4-AA metabolite was recrystallized from toluene. The purified 4-AA was identified with melting point measurement. The 4-AAA metabolite was synthesized by acetylation using acetic acid anhydride, the 4-FAA one was prepared in toluene by formylation of 4-AA, and the 4-MAA was synthesized through the KOH-catalyzed decomposition of dipyrone. Identification and purity of the compounds were checked by several methods including thin layer chromatography (TLC), nuclear magnetic resonance (NMR) spectroscopy, Fourier transformation infrared spectroscopy (FT-IR) and melting point determination. TLC was performed on 60 F254 type silica gel plates. Melting points were determined in a Boetius apparatus. The $^{1}$H- and $^{13}$C-NMR spectra were recorded in $CDCl_3$ or DMSO-$d_6$ solution at room temperature at 399.9 MHz ($^{1}$H) and 100.5 MHz ($^{13}$C), with the deuterium signal of the solvent as the lock and the residual solvent signal as an internal standard.

Chemical shift values (δ) were given in part per million. FT-IR spectra were recorded by using Nicolet Impact 400 spectrophotometer in KBr pellets. In all cases, for each substance the results were identical to the data reported in the literature.

## 4. Instrumentation

### 4.1. SPE-HPLC-ESI-Q-MS

To measure the metabolite content of the samples collected in February 2011, an HPLC-MS method was developed at the set-up operating at the Institute of Biochemistry and Medical Chemistry of the University of Pécs consisting of an HPLC equipped with a Dionex UV diode array detector and a Finnigan AQA mass spectrometer. The HPLC separation was performed using a Synergy-Hydro-RP 80 A C18 (150 × 2.0 mm × 4.0 μm) column and an AQ C18 Security Guard (40 × 2.0 mm) cartridge. The measurements were carried out at room temperature. The separation of the metabolites was achieved using the following multi-step gradient method: as eluent A a 10 mmol/L ammonium acetate buffer (pH=5.6) containing 2.5 V/V% methanol was applied (pH adjusted with acetic acid). As eluent B, a 10 mmol/L ammonium acetate buffer (pH=5.6) containing 70 V/V% methanol was used. Starting from 100 % eluent A composition, the composition

of eluent B was increased to 30 % within one minute. In the next 15 minutes, the composition of eluent B was further increased to 80 % and after that it was maintained for one minute. Then, the composition of eluent B was increased to 100 % and hold for another six minutes. Finally, the gradient composition of eluent A was raised to 100 % in 10 minutes and hold at 100 % for one minute more. The flow rate was 0.3 mL/min. The MS measurements were performed in electrospray ionization (ESI) mode. Spectra were recorded from m/z 100 to 300 by 2 scan/s. Positive ions of the metabolites were monitored during the analysis.

**4.2. SPE-HPLC-ESI-Q-TOF-MS/MS**

The liquid chromatographic separation was carried out in this case also on the HPLC column and SPE cartridges described in *Section 4.1.*. However, unlike to the method described in *Section 4.1.*, column thermostation could be achieved, so the measurements were performed at 40 °C. A Waters Acquity HPLC system was used for the HPLC separation. A multi-step gradient method was applied to separate the metabolites. An aqueous solution of 5 V/V% acetonitrile and containing 0.05 V/V% formic acid was used as eluent A, while 100 % methanol as eluent B. The flow rate was 0.4 mL/min. The initial composition of the mobile phase was 95% eluent A and

5 % eluent B. Then, the composition of eluent B was increased to 90 % in 1.5 minutes and was hold at 90 % for another 1.5 minutes. At the end of the gradient program, the composition of eluent A was increased to 95 % initial level in 0.1 minutes. The injection volume was 2.0 μL. The MS detection of metabolites was performed by using a Waters Micromass Q-TOF-Premier MS instrument in ESI mode. Metabolites and their fragments were detected in positive ionization mode. Data were collected between m/z 150 and 400. Exact masses of molecules were determined using the software of the MS.

## IV Results

### 1. Comparison of wastewater treatment technologies

As expected, the concentration of all metabolites was higher in the influent wastewater than in the treated, effluents ones. Generally, all investigated metabolites could be detected in the low μg/L range in the wastewater samples from all wastewater treatment plants, however in some cases the concentration of 4-MAA fell under the detection limit both in influent and effluent waters. For the characterization of the efficiency of treatment technologies, removal extent of the metabolite can be used, expressed as the concentration ratio of

the given metabolite in the effluent and influent wastewater in percentage. In the case of the North-Pest Wastewater Treatment Plant using activated sludge technology, the removal efficiency for the 4-AAA metabolite was 65% and 77 % in summer and autumn, respectively, while in winter and early spring more than 95 % was achieved. Besides the bacteria population characteristic to a certain wastewater treatment technology, the metabolite degradation depends on the water temperature and dissolved oxygen concentration. In wintertime, the dissolved oxygen concentration is higher due to the low ambient temperature and activated sludge wastewater treatment performed in aerated opened basins, which favors the proliferation of psychrophilic bacteria. By applying the same calculation for the samples collected at Telki, where fixed biofilm covered reactors are applied, a uniform removal efficiency of 80–94 % was obtained for 4-AAA independently from the sampling season. In Telki, due to the water aeration and recirculation system applied in this fixed biofilm reactor wastewater treatment plant the minimum and maximum air temperatures ranged 8 °C and 30 °C and the water temperatures ranged between 16 °C and 31 °C throughout the sampling period. Based on the above mentioned reasons, it was understandable that the removal

efficiency for 4-FAA was below 5 % in summer and autumn in North- and South-Pest Wastewater Treatment Plants. A modest increase (20–30 %) in the removal efficiency of 4-FAA was observed in winter and early spring of 2012. Another trend was observed for the removal efficiency of 4-FAA in terms of removal efficiency, which was 2–3 % in summer and winter, while 30–40 % in autumn and spring months. Based on these studies, it can be stated that 4-FAA is the most persistent metabolite.

## 2. Seasonal changes in the dipyrone metabolite concentration in influent wastewater samples from North-Pest Wastewater Treatment Plant

The concentration of 4-AA, 4-AAA and 4-FAA in the influent samples collected in the North-Pest Wastewater Treatment Plant was higher during the winter period (November 2011–February 2012) compared to the summer one (July and August 2011). The mean values showed 2.7 times, 1.4 times and 1.6 times higher concentration values for 4-AA, 4-AAA and 4-FAA, respectively. A similar trend was observed for the samples collected in autumn (September and October 2011). These data also clearly indicate a relationship between the increased use of the dipyrone containing drugs, as popular antipyretic and analgesic products and the winter season.

Moreover, the concentration level of 4-AA, 4-AAA and 4-FAA metabolites in the early spring season remained almost equal to that of the winter season.

**3. *Intra*day variation of the concentration of dipyrone metabolites in South-Pest Wastewater Treatment Plant**

For the samples collected every 6 hours, a peak value in the concentration of 4-AA, 4-AAA and 4-FAA was registered in the samples collected at noon. The samples collected at 06:00 AM were considered as the reference point for further calculations. An increase for 4-AA, 4-AAA and 4-FAA metabolites amounting to 46 %, 72 % and 73 %, respectively, was observed in the concentration of the samples collected at noon in comparison to those collected 6 hours before. A similar conclusion could not be drawn for 4-MAA due to its low concentration close to the limit of quantification characterized by high relative standard deviation, as well.

**4. Effect of chlorination on metabolite removal**

The effect of chlorination could only be investigated in samples collected at the South-Pest Wastewater Treatment Plant, as disinfection of the already biologically treated water is only applied there with 15 mg/L $Cl_2$ due to the vicinity to suburban resort area of Ráckeve. Taking into consideration the average concentration of three samples collected monthly

before and after chlorination, a very similar removal rate was calculated for 4-AAA and 4-FAA metabolites supposing a constant input of wastewater load to the wastewater treatment plant during the sampling period. The average of the removal efficiency was further 17 % and 15 % for 4-AAA and 4-FAA, respectively. Similar estimation could not be made for 4-AA and 4-MAA, since their concentration in the samples was close to the limit of quantification in several cases.

## V Conclusions, new scientific results

1. For the first time in Hungary, the four main metabolites of dipyrone (4-AA, 4-AAA, 4-FAA, 4-MAA) were determined in the influent and effluent water of three different wastewater treatment plants. Among the investigated dipyrone metabolites, the concentration of 4-AAA and 4-MAA were the highest (1.38-2.34 μg/L) and the lowest (0.007-0.089 μg/L) in the influent water samples in Budapest, respectively.

2. The concentration of the four investigated metabolites showed a seasonal variation in influent waters of the wastewater treatment plants. In the autumn-winter periods, a 38-161 % increase was observed in the concentration, which

can be explained by increased drug taking habits in that season.

3. The *intra*day variation of the concentration of dipyrone metabolites in the influent water of the wastewater treatment plants was significant. Values measured in the samples collected at noon were 46-75 % higher than in the samples collected at 6:00 AM.

4. The 4-FAA metabolite is proven to be persistent. Its removal efficiency was 0.7-37 % and 2-40 % in the North Pest Wastewater Treatment Plant using activated sludge technology and in the wastewater treatment plant at Telki applying fixed biofilm technology, respectively.

5. For both technologies, the 4-AAA metabolite had the highest removal efficiency, 80 % for the activated sludge wastewater treatment technology, while in the case of fixed biofilm technology 96 %.

6. In the summer season, the removal efficiency of the conventional (activated sludge) wastewater treatment plant was lower by 30 % for 4-AAA metabolite than in the cold season.

7. Chlorination (15 mg/L $Cl_2$) used as disinfection method for biologically treated wastewaters increased the removal efficiency of the 4-AAA and 4-FAA metabolites by an average of 15 %.

## VI Publications

**1.** Szabó Z, Szoboszlai N, Jámbor É, Gulyás G, Lóránd T, Ohmacht R, Záray G, Mihucz VG. (2013) Determination of four dipyrone metabolites in Hungarian municipal wastewater by liquid chromatography mass spectrometry. *Microchemical Journal, 107:* 152-157. IF: 2.850

**2.** Szabó Z, Szoboszlai N, Frigyes D, Záray Gy, Mihucz VG. (2014) Monitoring of four dipyrone metabolites in communal wastewater by solid phase extraction liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis, 90:* 58-63. IF: 2.853

**3.** Szoboszlai N, Réti A, Budai B, Szabó Z, Kralovánszky J, Záray G. (2008) Direct elemental analysis of cancer cell lines by total reflection X-ray fluorescence. *Spectrochimica Acta Part B: Atomic Spectroscopy, 63:* 1480-1484. IF: 3.047

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