Biodegradation of pharmaceuticals by common microorganisms

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Abstract

The biodegradation of selected pharmaceuticals has been studied using common microorganisms, in order to understand their fate in the environment. The pharmaceuticals selected for this study are known micropollutants. Carbamazepine is a drug used extensively in the treatment of epilepsy. Sulfamethizole, sulfamethoxazole and trimethoprim are common antibiotics. The microorganisms used were *Rhodococcus rhodochrous, Pseudomonas putida, Pseudomonas fluorescens, Bacillus subtillis* and *Sphingomonas herbicidovorans*. Biodegradation has been observed for carbamazepine, sulfamethizole and sulfamethoxazole.

Keywords: biodegradation, carbamazepine, sulfamethizole, sulfamethoxazole, trimethoprim.

1 Introduction

The presence of pharmaceuticals in wastewater has been recognized as an increasing source of pollution in the last few decades [1–4]. Pharmacokinetic studies have shown that a large proportion of this pollution comes from feces and urine [5], but a significant amount is also probably due to the disposal of expired pharmaceuticals in toilets or garbage. Although wastewater is treated to remove BOD and pollutants, the typical methods employed today are not designed to remove these compounds. The result is poor removal efficiencies of pharmaceuticals [2, 6, 7] and an accumulation of drugs in the aquatic environment [8–11]. Some studies have also reported the presence of these contaminants in drinking water at the nanogram per liter level [12]. Many of these pharmaceuticals have a high bioactivity and can have an effect even at concentrations as low as few ng/L. Moreover, some drugs in the environment

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may be transformed, either photochemically or by the action of microorganisms, to compounds that have higher toxicity then their parent compounds. Some of the possible consequences of the presence of drugs in drinking water are an increase in cancer occurrence, the proliferation of anti-biotic resistant bacteria and a potential increase in toxicity due to mixture effects [8, 13]. These considerations make it imperative to elucidate the fate of pharmaceuticals in the environment in order to protect public health and the integrity of soil and water resources.

The aim of this research was to study the biodegradation of selected pharmaceuticals by common microorganisms in order to shed some light on the fate of the pharmaceuticals in the environment. The pharmaceuticals studied have been repeatedly found in the environment and are considered to be micro pollutants [1, 7, 8, 12]. Three antibiotics were studied: sulfamethoxazole, sulfamethizole and trimethoprim as well as one antiepileptic drug, carbamazepine. Pure cultures of microorganisms commonly found in soils were chosen to evaluate their potential to degrade the selected drugs. The microorganisms chosen were known to degrade similar molecules as the pharmaceuticals of interest or at least the same functional groups. The microorganisms used were *Rhodococcus rhodochrous*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Bacillus subtilis*, *Aspergillus niger* and *Sphingomonas herbicidovorans*. In the environment these pharmaceuticals are present in such small quantities they are not the primary substrate. So another primary carbon source was used in the experiments and the pharmaceuticals were co-substrates. This allowed for adequate growth of the microorganisms and minimized toxic effects due to high concentrations of the pharmaceuticals.

2 Materials and methods

2.1 Chemicals and microorganisms

Carbamazepine (CBZ), sulfamethizole (SMZ), sulfamethoxazole (SMX), trimethoprim (TMP), DMSO, formic acid and ammonium acetate were purchased from Sigma-Aldrich. Methanol HPLC grade, acetonitrile HPLC grade, water HPLC grade, phosphoric acid HPLC grade, EDTA, ammonium nitrate, Na_2HPO_4 and KH_2PO_4 were obtained from Fisher. Calcium chloride dihydrate and ferrous sulfate heptahydrate were bought from Acros whereas magnesium sulfate heptahydrate and D-glucose anhydrous were bought from A&C. BHI and yeast extract came from Becton, Dick and Comp. Microorganisms *Rhodococcus rhodochrous* (13808), *Pseudomonas putida* (12633), *Pseudomonas fluorescens* (13525), *Bacillus subtilis* (6051), *Aspergillus niger* (16888) and the *Sphingomonas herbicidovorans* (700291) were all purchased from ATCC.

2.2 Growth experiments

Growth experiments were conducted in shake flasks (500 ml) equipped with a foam cap in an incubator shaker at 26 °C and at 150 RPM. Each flask contained 100 ml of media and was autoclaved prior to experiments. For experiments

conducted with sulfamethizole, sulfamethoxazole and trimethoprim, shake flasks were covered with aluminum foil to prevent photodegradation of the compounds. Microorganisms were allowed to grow twice in the brain heart infusion (BHI) media, and then transferred 2 times in the minimum mineral salt media (MMSM). The microorganisms were then grown in MMSM with the pharmaceutical (an acclimation period) and, finally, transferred to flasks for the degradation experiments. Three controls were used: without drug, with drug but without microorganisms and with dead microorganisms. The flasks were weighed to allow for the compensation for water evaporation.

2.3 Media preparation

Brain heart infusion media was prepared by weighting 3.7 g of BHI in a shake flask with 100 ml of distilled water. Minimum mineral salts media was made to have a final concentration of 0.018 g/L of Na_2EDTA, 0.013 g/L of $FeSO_4$ hexahygrate, 0.013 g/L of $CaCl_2$ dihydrate, 0.25 g/L of $MgSO_4$ heptahydrate, 7.5 g/L of Na_2HPO_4, 5 g/L of KH_2PO_4, 5 g/L of NH_4NO_3 and 0.63 g/L of yeast extract Glucose was added separately at a concentration of 3 g/L.

Each pharmaceutical was studied separately. The stock solution of carbamazepine was prepared at approximately 100 ppm in distilled water and was autoclaved prior to inoculation as no degradation of carbamazepine was observed during autoclaving. Sulfamethoxazole and sulfamethizole stock solutions were prepared at 400 ppm in distilled water. Trimethoprim stock solution was prepared at about 12500 ppm in DMSO and kept under nitrogen. Sterilized filters were used to transfer these drugs into flasks.

2.4 Inoculation and sampling procedures

Inoculations were done in the laminar flow hood with sterilized equipment. In all experiments, 80 ml of the MMSM solution was added first. For CBZ, SMX and SMZ experiments, 10 ml of the stock solution and 10 ml of glucose solution were added to a flask followed by 1 ml of microorganisms in acclimated solution. For TMP experiments, 1 ml of the stock solution, 1 ml of acclimated microorganism solution and 10 ml of the glucose solution were added in a flask. For a control without drugs, the stock solution was replaced by the same amount of distilled water. In controls without biomass, distilled water replaced the microorganism solution.

Sampling was done under the laminar fume hood. Samples (2 ml) were taken from a flask and transferred to plastic centrifuge tubes. Samples were then centrifuged for 10 minutes at 10,000 RPM. The supernatant was filtered with 0.45 micron HPLC filters after which samples were ready to be analyzed by HPLC.

2.5 HPLC methods

HPLC analysis was performed using an Agilent 1100 HPLC with a variable wavelength detector. The method used for each pharmaceutical is described below.

2.5.1 Carbamazepine

An Eclipse XDB-CN 5μm (4,6x150 mm) column was used. Elution conditions were 70% of 25 mM ammonium acetate buffer adjusted to pH 3.0 with formic acid (solvent A) and 30% acetonitrile (solvent B). The flow rate was 1.0 ml/min and the detection wavelength was set to 220 nm. The injection volume was 25 μl and the temperature was kept at 20 °C. The total analysis time was 10 minutes.

2.5.2 Sulfamethizole and sulfamethoxazole

An Eclipse XDB-C18 5 μm (4,6x250 mm) column was used. Elution conditions were 70% of 25 mM NaH_2PO_4 buffer at pH 2.8 (solvent A) and 30% acetonitrile (solvent B) for 10 minutes, then 45% of B for 5 minutes followed by 55% of B for 1 minute and finally 95% of B for 14 minutes. The flow rate was 0.7 ml/min and the detection wavelength was set at 273 nm. The injection volume was 5 μl and the temperature kept at 40 °C. The total analysis time was 30 minutes.

2.5.3 Trimethoprim

An Eclipse XDB-C18 5 μm (4,6x250 mm) column was used. Elution conditions were 85% of a mixture of methanol/water (60:40) at pH 3.0 (solvent A), and water (solvent B) 15%. The flow rate was 1.2 ml/min and the wavelength was set to 230 nm. The injection volume was 5 μl and the temperature was kept at 35 °C. The total analysis time was 8 minutes.

3 Results and discussion

3.1 Limit of quantification (LOQ) and limit of detection (LOD)

LOD and LOQ are presented in table 1 and were calculated as appropriate. For the carbamazepine and sulfamethizole methods, the LOD was defined as $3S_{y/x}/m$, where m is the slope of the calibration curve and $S_{y/x}$ is the standard deviation on the regression. LOQ was defined as $10S_{y/x}/m$. For sulfamethoxazole and trimethoprim, a background equivalence correction (BEC) was applied. The BEC consisted of calculating the standard deviation of the signal of the blank at least 4 times at the retention time of the drug. The LOD and the LOQ correspond respectively to 3 and 10 times this standard deviation.

3.2 Carbamazepine degradation experiments

As reported in the literature, carbamazepine appeared to be a very persistent compound and thus not easily degraded. No toxic effects, such as slower growth, were observed at the concentrations tested. For experiments conducted with *Pseudomonas putida*, *Pseudomonas fluorescens*, *Sphingomonas herbicodovorans*, and *Bacillus subtillis*, no degradation was observed. The decrease in concentration measured was similar to the one observed in control samples. The decrease was attributed to the abiotic degradation of carbamazepine. Controls and samples followed the same trend to reach a

decrease in concentration of 5% or less. In these experiments, absorption by the microorganisms was not found to be significant. Metabolites were not observed in chromatograms of any of these experiments, which lasted 10 days for *Pseudomonas putida*, *Sphingomonas herbicodovorans*, and *Bacillus subtillis* and 14 days for *Pseudomonas fluorescens*. The length of the experiments was always sufficient for the microorganisms to reached and exceed the stationary phase. With all of these results, it was concluded that none of these microorganisms used carbamazepine as a co-substrate.

Table 1: LOD and LOQ of the analytical method.

Pharmaceutical	LOD (ppm)	LOQ (ppm)
Carbamazepine	0.36	1
Sulfamethizole	0.25	1
Sulfamethoxazole	0.25	1
Trimethoprim	0.65	2

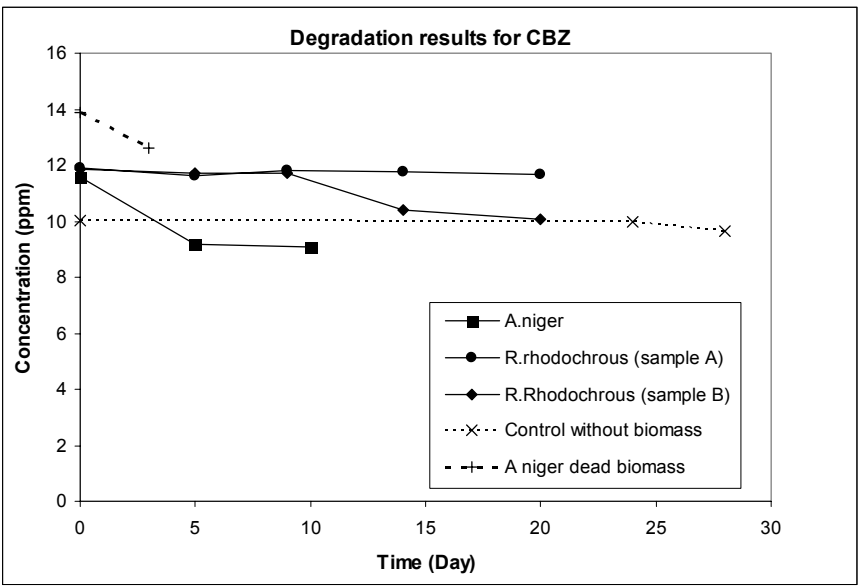

Figure 1: Degradation of carbamazepine by *Rhodococcus rhodochrous* and *Aspergillus Niger*.

Figure 1 shows a degradation experiment of carbamazepine with *Rhodococcus rhodochrous* and *Aspergillus Niger*. In the experiments with *Aspergillus Niger* a significant decrease in concentration was observed. The decrease was about 20%, although this included some absorption of carbamazepine by the microorganisms. The absorption by the dead biomass in the control was determined to be 10%. Overall there is still a decrease of 10%

due to biodegradation. In addition, this data is supported by the observation of a new compound as determined by the HPLC. Further analyses are underway to determine the structure of this proposed metabolite from the biodegradation of carbamazepine.

The experiments conducted with *Rhodococcus rhodochrous* were consistent with degradation of carbamazepine with *Aspergillus niger*. There was some variation in these values, which showed as much as 15% degradation. However, the degradation observed in the control was only about 4%. Again chromatograms of the samples have shown a metabolite at low concentrations but this has not been identified.

3.3 Sulfamethizole and sulfamethoxazole degradation experiments

Experiments with SMX and SMZ were only conducted with *Rhodococcus rhodochrous*. These experiments are illustrated in figure 2. For both drugs, decreases in concentration in the order of 20% were observed relative to about 5% in the controls. The controls with biomass showed low absorption of SMX. The growth rate of the microorganisms was a little bit slower in the presence of the antibiotic compared to the controls.

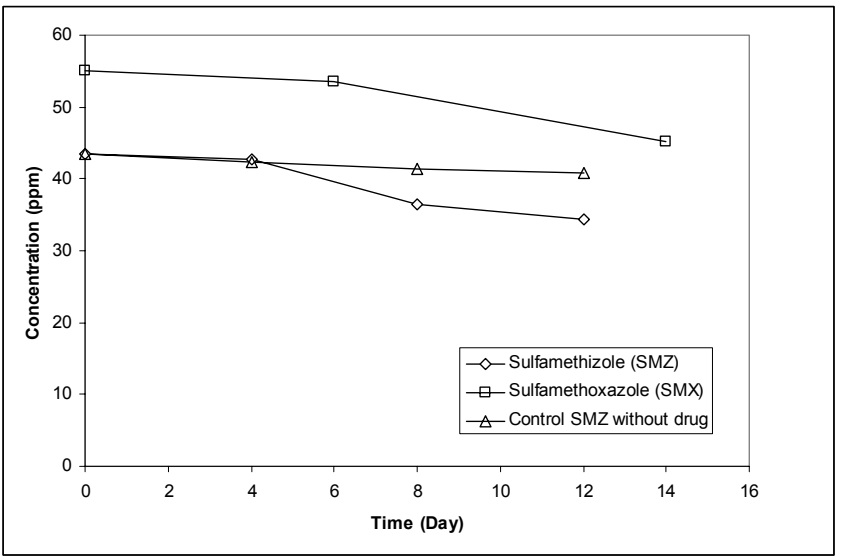

Figure 2: Degradation of sulfamethizole and sulfamethoxazole by *Rhodococcus rhodochrous*.

Figure 3 shows the HPLC chromatograms obtained at the beginning and at the end of a degradation experiment. Several new peaks were observed in the chromatograms of the degraded samples. However, these have not been identified yet.

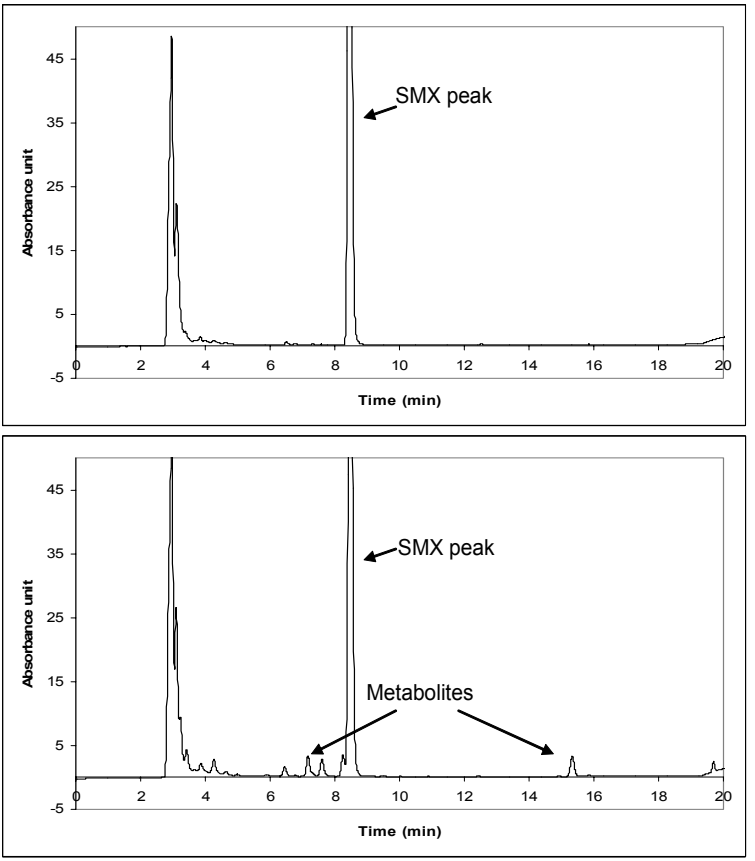

Figure 3: HPLC chromatograms evolution in degradation experiment of sulfamethoxazole.

3.4 Trimethoprim degradation experiments

Among all the pharmaceuticals tested, TMP was definitely the most resistant to biodegradation. Experiments were conducted with *Pseudomonas putida*, *Rhodococcus rhodochrous* and *Aspergillus niger*. In every experiment, no degradation was observed. The concentration of the drug was ten times higher then the level used in CBZ experiments and may imply that if there is any degradation it is so small that it could not be detected in HPLC analysis. Although the concentrations were high compared to CBZ experiments, no toxic effects were observed during the growth of the microorganisms. Also, no changes in the chromatograms were observed.

4 Conclusion

The results obtained for all the degradation experiment are summarized in table 2. The dash line means that the experiment has to be done. The drugs in

this study were present as co substrates in the presence of a primary carbon sources. Among the pharmaceuticals tested trimethoprim was found to be the totally non-degradable. However, two different microorganisms (*Rhodococcus rhodochrous* and *Aspergillus niger)* were able to cause at least some biodegradation of the other pharmaceuticals considered. In several of the experiments small amounts of suspected metabolites were observed but none of these have been identified yet.

Table 2: Summary of results obtained from degradation experiments.

Compound/ Microorganism	CBZ	SMZ	SMX	TMP
Rhodococcus rhodochrous	15%	14%	20%	0%
Aspergillus niger	10%	---	---	0%
Sphingomonas herbicidovorans	0	---	---	---
Bacillus subtillis	0	---	---	---
Pseudomonas fluorescens	0	---	---	0
Pseudomonas putida	0	---	---	---

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