Toxicological evaluation of carbamazepine active pharmaceutical ingredient with *Lepidium sativum*, *Daphnia magna* and *Vibrio fischeri* toxicity test methods

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**Abstract**

Pharmaceuticals have been found extensively within an aquatic environment. Carbamazepine was one of them that was found at the highest frequency and amounts. The persistence of these micropollutants in the environment is of concern because of a combination of characteristics, which includes the toxicity of humans and animal health. In this study, acute toxicity from carbamazepine, which is regularly used in anti-epileptic pharmaceuticals, was investigated using *Lepidium sativum* (*L. sativum*), *Daphnia magna* (*D. magna*), and *Vibrio fischeri* (*V. fischeri*) toxicity test methods. These different toxicity test methods were used and their sensitivities were compared. The results obtained from all experiments were evaluated according to the applied method. Accordingly, electrical conductivity (EC₅₀) (the 50% effect concentration) values ranged from 9.53 to 94.39 mg/L. The test results were expressed as “toxic unit” (TU) which were calculated as 1.05 for *L. sativum*, 10.49 for *D. magna*, and 2.7 for *V. fischeri*. According to the TU values, sensitivities were *D. magna* > *V. fischeri* > *L. sativum*, respectively. The most sensitive values were obtained from *D. magna* toxicity test method.

**Keywords:** Acute toxicity; Carbamazepine; Pharmaceutical; Toxicity tests; Toxic unit

1. Introduction

The amount of pharmaceuticals reaching and affecting the environment has led to a growing concern in recent years, particularly with regard to their potential risks to aquatic environments [1]. The occurrence of pharmaceutical products (“micropollutants”) in the aquatic environment, especially urban and hospital wastewaters, effluents from water and sewage treatment plants, surface waters, and some drinking water, is being monitored in developed countries. These micropollutants are partially or wholly biotransformed into hydrophilic metabolites within an organism and then pass into the environment [2,3]. The main concern is related to their persistence within the environment because of a combination of characteristics, such as toxicity to human and animal health. In addition, many residual pharmaceuticals are resistant to conventional drinking water and wastewater treatments therefore, they are only partially removed from these treatment systems [4,5].

In the late 1990s, pharmaceuticals and personal care products (PPCPs), which are widely used in daily life, was identified as a potential risk to wildlife [6]. These PPCPs include several chemical classes. Pharmaceuticals are used primarily to prevent or treat human and animal diseases, whereas personal care products are used to improve the quality of daily life and include products such as moisturizers, lipsticks, shampoos, hair colors, deodorants, and toothpaste. Pharmaceuticals can be introduced into the environment in several ways during or after production or consumption [7] and their use and environmental prevalence increase annually for various reasons, such as...
the expansion of medical treatments, greater availability of drugs worldwide, affordable prices, population growth, and population aging (in some countries) [8]. Pharmaceuticals are used for both therapeutic and veterinary purposes. Eventually, it is released into the environment. The concentration of PPCPs in aquatic environments usually ranges from ng/L to a few μg/L [9]. These PPCPs are generally designed to have specific physiological effects on humans and other animals; however, they can also negatively affect aquatic organisms. Active pharmaceutical ingredients (APIs) are detected throughout the environment in water, soil, sediment, and sludge as well as in drinking water in some countries [10]. Although the presence of pharmaceuticals does not always harm the environment or human health, concerns are growing about their chronic effects on biodiversity, including antimicrobial resistance and the endocrine-disrupting effects on fish [11].

Carbamazepine is one of the most widely used anti-convulsants in the treatment of a tonic–clonic convulsive crisis, which selectively depresses responses in the central nervous system, without causing harm or respiratory depression [12]. Many studies have noted the presence of anti-anxiety and anti-epileptic drugs in environmental samples. Carbamazepine (1,400 ng/L) was detected in surface waters in Aachen-Soers, Germany. [13]. There is also a report in Western Australia on the contamination of reclamation waters with carbamazepine (at 1,000 ng/L) [14]. In Catalonia (Spain), 113 and 175 ng/L carbamazepine were detected in the influent and effluent samples, respectively, from sewage treatment plants [15]. Pharmaceutical compounds can accumulate biologically and then change the physiological and reproductive functions of aquatic organisms [16].

Many of these micropollutants cause significant ecotoxicological concerns, especially when they are components of a complex mixture [17]. The main focus of ecotoxicological research lies on the identification of substances that pose a high risk for the environment, but the limited capacity of testing facilities can result in a tremendous increase in the number of present and future micropollutants; therefore, fast and simple testing methods that can generate reliable and meaningful data are desperately needed to assess environmental risks from PPCPs. The ability to test for toxicity has increased steadily in recent years and is a useful bioassay for environmental risk. Toxicity tests on water pollution contaminants are typically conducted by testing the effects of a single pollutant on a variety of organisms, such as plants, (bacteria, algae, daphnia, and fish). The toxicity effects are measured in terms of, for example, immobility or mortality Daphnia magna (D. magna), decreased luminescence Vibrio fischeri (V. fischeri), growth inhibition Lepidium sativum (L. sativum), or reproduction inhibition in certain test organisms, depending on the time of exposure and the concentration or dilution. L. sativum is an economically viable plant to use in a toxicity test. V. fischeri is a gram-negative, rod-shaped bacterium with bioluminescent properties that is sensitive and fast, easy to control and has a wide range of applications. Tests on these three species are useful because the species represent different trophic levels. The purpose of this study was to compare the sensitivities and toxicity levels of PPCPs in plants and aquatic life using these representative species.

2. Material and methods

2.1. Working samples

When similar studies [18–20] that evaluated the toxicity of a single parameter were examined, a synthetic sample was preferred to prevent interference from other parameters in real wastewater. Synthetic pharmaceutical solutions were prepared at specific concentrations (e.g., 1,000, 500, 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.91, and 1.95 mg/L) as working samples. “Carbamazepine (CAS No. 298–46–4)” is an anti-epileptic drug solution and the active ingredient that was tested. Its chemical structure is provided in Fig. 1. The pH was measured using the Jenway 3010 pH-meter; electrical conductivity (EC) was read using a Hach portable case conductivity meter (Loveland, CO, USA). Dissolved oxygen was measured using a portable dissolved oxygen meter (Hach). The physicochemical properties of carbamazepine are provided in Table 1.

2.2. Bioassay tests

The selected test protocols are provided in Table 2. L. sativum, a terrestrial plant, was selected to represent the trophic level of producers. V. fischeri, a bacterium, was selected to represent the decomposers, D. magna, a crustacean, was selected to represent the consumers.

2.2.1. Lepidium sativum toxicity test

The L. sativum toxicity test was performed using 3 controls and 10 different concentrations of a synthetic pharmaceutical solution. Two pieces of filter paper were placed into Petri dishes and 5 mL deionized water was added to the control Petri dishes. Each of the 25 L. sativum seeds was equally distributed into each petri dish and covered. Each of the different concentrations of the pharmaceutical samples was placed into three Petri dishes. The experimental procedure was repeated for each of the different concentrations. The Petri dishes were cover and placed in the dark for 48 hours.

Fig. 1. Chemical structure of “carbamazepine”.

Table 1

<table>
<thead>
<tr>
<th>Active pharmaceutical ingredient</th>
<th>Carbamazepine</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.7</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>24</td>
</tr>
<tr>
<td>Electrical conductivity (μS/cm)</td>
<td>514</td>
</tr>
<tr>
<td>Dissolved oxygen (mg/L)</td>
<td>8.8</td>
</tr>
</tbody>
</table>
and incubated for 72 h, at 25°C. After 72 h, the number of roots, and hypocotyls and the root heights grown from the *L. sativum* seeds were counted and measured. The average and mean values of root length were measured in the control seeds at the end of test time, and the percentage of height inhibition and the 50% effect concentration (EC50) values were determined and compared.

2.2.2. *Daphnia magna* toxicity test

The *D. magna* toxicity test was conducted according to standard test procedures (OECD, 2004; Test No: 202) [21]. Each increasing concentration of the synthetic samples was placed in test containers. For each sample concentration, five *D. magna* were placed in the cells of the test plate. A control group was created with five *D. magna* in each control group. At the end of the 24 and 48 h incubation period, immobilized and dead *D. magna* from each experimental vessel were counted. Accordingly, EC50 values were calculated and a graphical interpolation of the mortality and immobility inhibition rate was created (Fig. 3).

2.2.3. *Vibrio fischeri* toxicity test

*V. fischeri* luminescent bacteria were stored in a freezer until use. They were then kept in a water bath for 2 min before the experiment to reach room temperature. Reactivation solution at 15°C was poured onto the bacteria and remained for 15 min to first activate the bacteria. Drug samples at different concentrations were prepared in a series of dilutions directly in the test vials of the initial concentration with the aid of diluent for each of the samples. The prepared bacterial solution was transferred to a series of cuvettes and the drug solutions on which the toxicity tests were performed were transferred to another series of cuvettes. The light emission intensity (I0) was measured before each bacterial solution was transferred onto the solution containing the drug according to the test procedures. *V. fischeri* luminescence was measured at 15°C and 490 nm. Any decreasing light emission from this species of bacteria in the presence of toxic substances indicated a toxic effect. The results are expressed as the concentration at which 50% of light emission (EC50) disappears at 5 (I5) and 15 (I15) min (Fig. 4) [22].

3. Results and discussion

3.1. *Lepidium sativum* toxicity test results

Toxicity to *L. sativum* was determined at the end of the 72 h test period for different concentrations of carbamazepine API. The EC50 value of the samples; and percent inhibition were calculated and a calibration curve corresponding to the concentration values was generated (Fig. 2). Toxic unit (TU) values were determined according to the calculated EC50 values.

The average root and hypocotyl lengths were compared. The percent inhibition of the root lengths in the drug

![Fig. 2. Percent inhibition of roots and hypocotyls of *Lepidium sativum* by the concentration of carbamazepine.](image)
samples was higher than that of hypocotyl lengths. Thus, the hypocotyls were not as sensitive to the drug as the roots.

3.2. *Daphnia magna* toxicity test results

*D. magna* toxicity test results were determined at the end of the 24th and 48th hour test period for carbamazepine API. The EC$_{50}$ values of the samples; and the percentage of mortality and immobility inhibition values are presented on the calibration curve shown in Fig. 3. TU values were determined according to the calculated EC$_{50}$ values.

The effects of different concentrations of carbamazepine on *D. magna* after exposure to the drug for 24 and 48 h are shown in Fig. 3. The percentage of the effects of the carbamazepine samples after 48 h was higher than that after 24 h.

3.3. *Vibrio fischeri* toxicity test results

*V. fischeri* toxicity test results were determined at the end of 5 and 15 min for different concentrations of carbamazepine API. The EC$_{50}$ value and the percent inhibition values of the samples were recorded on a calibration curve corresponding to the concentration values. TU values were determined according to calculated EC$_{50}$ values.

The effects on *V. fischeri* exposed to different concentrations of carbamazepine after 5 and 15 min were compared (Fig. 4). The effects after 15 min were greater than those after 5 min.

3.4. Assessment of the TU

Based on the EC$_{50}$ values obtained from our toxicity tests, TU values were calculated according (Eq. (1)). According to the acute toxicity classification system reported by Personee et al. [23], toxicity was determined as follows: class I (TU = 0) “no acute toxicity” class II (0 < TU < 1) “slightly toxic” class III (1 < TU < 10), “toxic”, and class IV (11 < TU < 100), “very toxic”. The TU values of different bioassays of drug samples are shown in Table 3.

\[
TU = \left( \frac{1}{EC_{50}} \right) \times 100
\]  

(1)

In this study, for synthetic wastewater containing carbamazepine API, pH, temperature, EC, and dissolved oxygen values were measured as 7.7, 24°C, 517 μs/cm, and 8.8 mg/L, respectively. These physicochemical parameters did not influence growth, immobility or mortality, and luminescence during the toxicity tests. The EC$_{50}$ concentration value obtained for carbamazepine for *D. magna* was 9.53 mg/L at the end of 48 h and for *V. fischeri* was 36.1 mg/L at the end of the 15 min. Kim et al. [24] have found that the EC$_{50}$ value is 45.8 mg/L for *V. fischeri*. Santos et al. [1], have found that the EC$_{50}$ value is 12.7 mg/L for *D. magna* at the end of the 48 h. When these results were compared, the carbamazepine API results were compatible and significant.

![Fig. 3. Effect of different concentrations of carbamazepine on *Daphnia magna* at 24 and 48 h.](image-url)

![Fig. 4. Effect of carbamazepine on *Vibrio fischeri* after exposure for 5 and 15 min.](image-url)
Table 3
Toxic units (TU) for “carbamazepine”

<table>
<thead>
<tr>
<th>Carbamazepine</th>
<th>EC50 (mg/L)</th>
<th>TU</th>
<th>Assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepidium sativum</td>
<td>94.39</td>
<td>1.05</td>
<td>Toxic</td>
</tr>
<tr>
<td>Lepidium sativum</td>
<td></td>
<td>0.52</td>
<td>Slightly toxic</td>
</tr>
<tr>
<td>Daphnia magna (24 h)</td>
<td>10.74</td>
<td>3.67</td>
<td>Toxic</td>
</tr>
<tr>
<td>Daphnia magna (48 h)</td>
<td>9.53</td>
<td>10.5</td>
<td>Toxic</td>
</tr>
<tr>
<td>Vibrio fischeri (5 min)</td>
<td>62.5</td>
<td>1.5</td>
<td>Toxic</td>
</tr>
<tr>
<td>Vibrio fischeri (15 min)</td>
<td>36.1</td>
<td>2.7</td>
<td>Toxic</td>
</tr>
</tbody>
</table>

4. Conclusion

In this study, acute toxicity of carbamazepine API, a commonly used pharmaceutical, was evaluated using representative model types of L. sativum, D. magna, and V. fischeri. These results indicated that the drug has toxic effects. Three different toxicity test species were used and their sensitivities to the drug were compared at different concentrations of the drug.

The TU values are presented, respectively, for the experiments, as D. magna > V. fischeri > L. sativum. The most sensitive of the three species used was D. magna. This study showed a negative effect on the environment of a specific pharmaceutical drug found in wastewater. Pharmaceutical residues have been identified in wastewater, groundwater, drinking water, soil, and sediment. The species used for these studies represent different trophic levels; therefore, these toxicity tests are very important methods by which to determine the effect of specific drugs on the aquatic environment.

References