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Original Article

Diazepam TiO_2 Photodegradation along with Metabolites Obtained from the Kinetic Study in Sludge

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ABSTRACT

This study aims to show the stability of diazepam, a member of the benzodiazepines and psychoactive drug, in the environment. Two experiments were carried out: biological degradation by using activated sludge and advanced oxidation processes (AOPs). AOPs process was proposed and applied. TiO_2, used as catalyst, has two important properties: high photo-catalytic activity and low cost. Photodegradation of diazepam was much faster under light irradiation in the presence of TiO_2 (half-life = 6 hours) than under Suntest irradiation (half-life = 34 hours). Considering that the photo-catalytic process started after the biological treatment on the water body containing a very little amount of contaminants, the removal of this pharmaceutical compound was quite complete and the degradation products were detected below the legal limit.

Keywords: diazepam, advanced oxidation processes (AOPs), photolysis and photo-catalysis

INTRODUCTION

Benzodiazepines (**Fig. 1 (1)**) have received recent consideration. A most known member of the benzodiazepines is diazepam (**Fig. 1 (2)**) (7-chloro-l-methyl-5-phenyl-1,4-benzodiazepin-2-one). Diazepam is considered as anticonvulsant, anxiolytic, sedative and muscle relaxant [1–3]. It is used to relieve anxiety, muscle spasms, and seizures and to control agitation in both adults and children [1].

Most of the substances swallowed undergo transformations in the human body with consequently releasing significant amounts of a variety of degradation products into the aquatic environment. These substances can be further transformed during sewage treatment. As all contaminants, pharmaceutical compounds are subjected to biotic and/or abiotic degradation. The degradation by-products are also of major concern, because they may have toxicity similar to or higher than the parent compounds [4–6].

Elucidation of biodegradation pathways and identification of transformed products of diazepam are of crucial importance in understanding contaminant's fate in the aquatic environment. It is well documented that diazepam is very slightly soluble in water, but it is soluble in alcohol [7].

The use of specialized and accurate analysis like liquid chromatography-mass spectrometry (LC-MS) has become one of the preferred techniques for analyzing the behavior of different contaminants occurring in the environment. For this reason LC-Fourier-transform ion cyclotron resonance mass spectrometer (FTICR MS) analysis has been used to identify the degradation products.

Many studies are performed on diazepam behavior especially in human bodies. This compound is metabolized in human liver via the cytochrome P450 enzymes, it has elimination half-life of 20 − 100 hours, and from the mother molecule it gain several pharmacologically active metabolites (**Fig. 2**) [8–11].

Its main active metabolite is desmethyl diazepam (nordiazepam (**3**)), which has an elimination half-life of 36 − 100 hours [7]. Other active metabolites are temazepam (**4**) and andoxazepam (**5**) (**Fig. 2**) which have elimination half-lives of 8 − 22 and 4 − 15 hours, respectively [11]. These metabolites, along with the parent compound are conjugated with glucuronide and are excreted primarily in the urine.

Human metabolism studies have shown that 30% of this pharmaceutical, taken by patient, is active as parent compound, 12% as nordiazepam, 15% as temazepam and 32%

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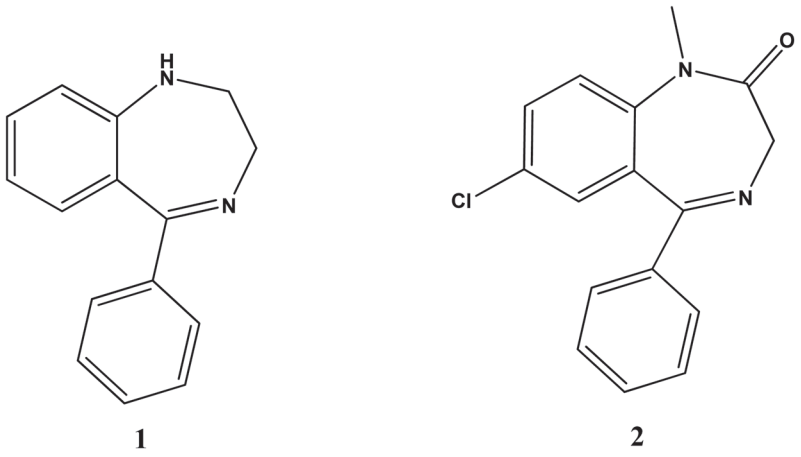

Fig. 1 Chemical structures of (**1**) benzodiazepineand and (**2**) diazepam.

as oxazepam [8–13]. The remaining 11% is unidentified to date [14]. As shown in **Fig. 2**, diazepam metabolism is mediated by a number of P450 enzymes and it occurs with N-dealkylation and a C3-hydroxylation [8,14–17].

Upon excretion of this drug, it reaches the wastewaters and thus, its presence becomes a problem for the environment.

Advanced oxidation processes (AOPs) [16] constitute an alternative to filtration methods in removing organic matter from wastewater. The mechanism of AOPs is the generation of highly reactive free radicals such as hydroxyl radical (·OH) which is effective in destroying organic chemicals. Photo-catalytic oxidation is one of the most effective clean technologies able to reduce/remove contaminants from wastewater and drinking water [17]. The mostly used photo-catalyst is TiO_2. This semiconductor can be used in a wide pH range, being able to produce electronic transitions by light absorption in the near ultraviolet range. TiO_2 is also characterized by its low cost, its high photo-catalytic activity, and its resistance to photo-corrosion [16–18].

The ·OH radicals formed on the illuminated semiconductor surface are very strong oxidizing agents with an oxidation potential of 2.8 V. These can easily attack the adsorbed organic molecules or those located close to the surface of the catalyst, thus, at the final stage, leading to their complete mineralization (**Fig. 3**).

Our interest in this study is to show the behavior of diazepam after TiO_2 photodegradation along with the identification of diazepam metabolites which were obtained from the kinetic study in sludge.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade:

- Diazepam was obtained from Sigma Aldrich (Steinheim, Germany). with 99% purity, and was used as received;
- Titanium dioxide, TiO_2 P-25 (anatase/rutile = 3.6/1), surface area 10 m^2/g, non-porous) was acquired from Degussa (Frankfurt, Germany);
- Deionized water, methanol and acetonitrile (both HPLC grade) were purchased from Sigma Aldrich (Steinheim, Germany).

Sample enrichment and purification

For sample enrichment and purification, the cartridges used for solid phase extraction were Oasis® HLB (200 mg, 6 mL) from Waters Corporation (Milford, USA). The syringe filters of 0.45 µm pore size were purchased from Pall Corp (Glen Cove, USA) were used. Samples were shaken using Big Bill, (Barnstead/ Thermolyne, Temecula, USA).

Activated sludge

Activated sludge used in stability test was obtained from the wastewater treatment plant (WWTP) located at Birzeit University-Palestine and was described in detail elsewhere [19].

Photo-chemical and photo-catalytic experiments

Photo-chemical and photo-catalytic experiments were conducted by using Suntest CPS solar simulator (Heraeus Instruments, Hanau, Germany) equipped with a xenon lamp, with a temperature sensor and a water-cooling circuit. The xenon lamp with stable borosilicate UV filter (Atlas Material

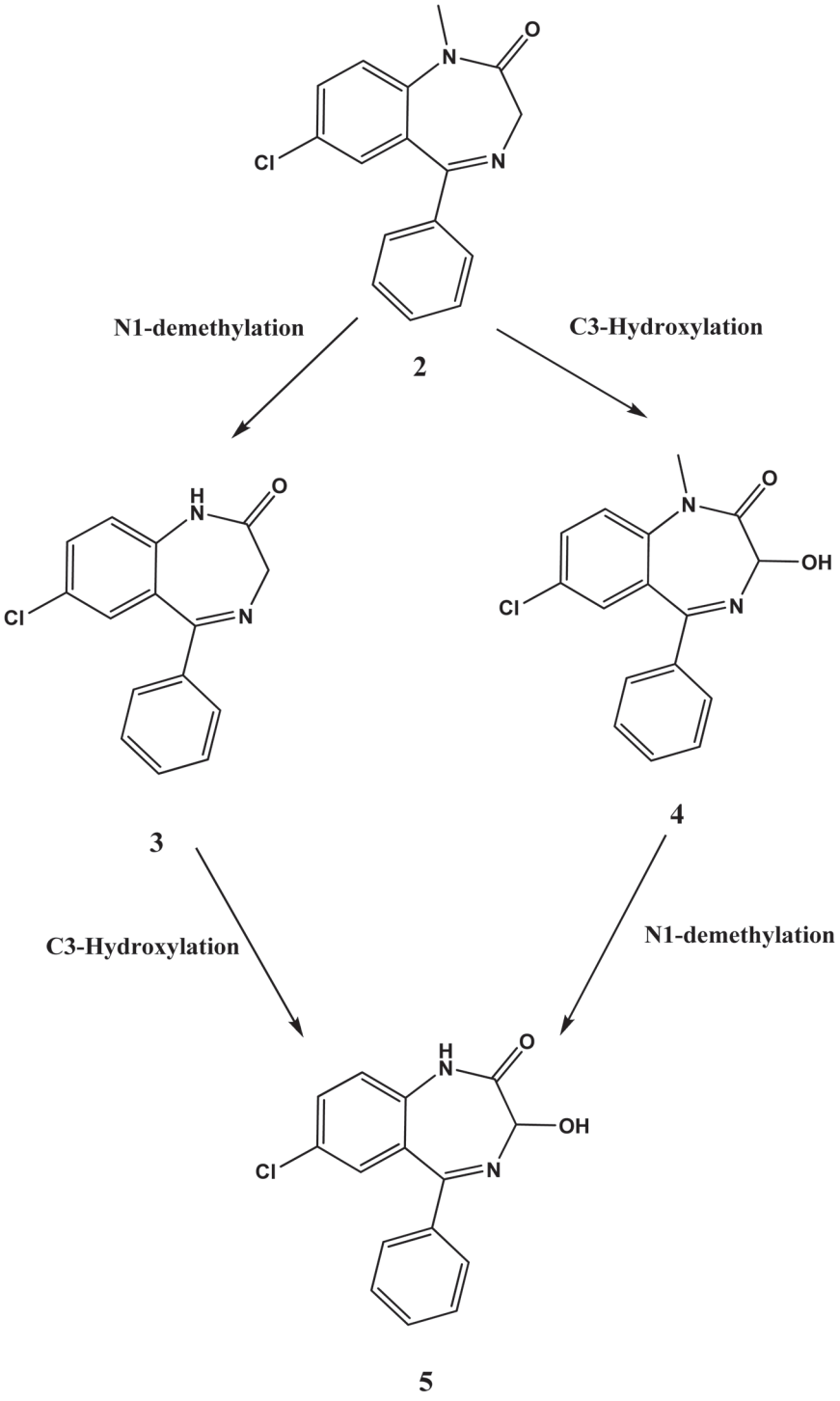

Fig. 2 Metabolic pathways of (**2**) diazepam to (**3**) nordiazepam, (**4**) temazepam, and (**5**) oxazepam in human liver.

Testing, Sanary, France) delivers a light emission spectrum similar to that of the sun. The range of wavelengths that are passed through the filter should be between 390 nm > λ > 340 nm.

A stock solution (100 mg/L) of diazepam pure standard in water was prepared and kept in the dark at +4°C. Calibration and working solutions of diazepam were prepared when used by dilution from stock solution.

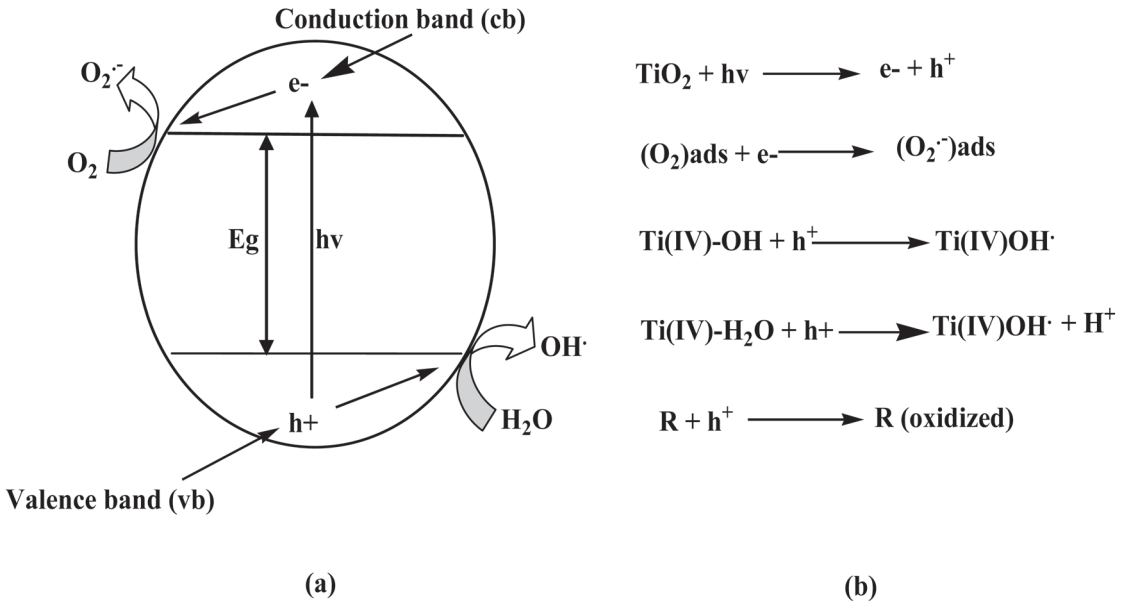

Fig. 3 (a) TiO_2 solution interface under UV-illumination. (b) Reactions on the TiO_2 surface under UV-illumination.

Analytical methods

Kinetic behavior of diazepam was performed by using a high performance liquid chromatograph (HPLC) Waters 2695 equipped with a photodiode array. Data acquisition and control were carried out using Empower™ software (Waters, Milford, USA). Chromatographic separation was achieved with a Purospher Star RP-18 endcapped column (125 mm x 2.0 mm, particle size 5 µm) preceded by a C_{18} guard column (4 x 4, 5 µm), both supplied by Merck (Darmstadt, Germany). A pH meter HM-30G (TOA electronics™, Heidelberg, Germany) was used to control the pH value of samples.

For the HPLC, the mobile phase was: acetonitrile: water (1:1; v/v); flow rate of 1.0 mL/min and the UV detection was set at a wavelength of 230 nm. Acrodisc® syringe filters with GHP membrane (hydrophilic polypropylene 0.45 µm porosity) from Waters were always used for all analytical filtration requirements [18,20–21].

All experiments were performed using a Surveyor LC system coupled to a hybrid LTQ-FTICR (7-Tesla) mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), equipped with a 20-W CO_2-laser (Synrad, Mukilteo, USA; 10.6 mm). LC separation was performed at ambient temperature on a Discovery C18 column, 250 × 4.6 mm inner diameter, 5 µm particle size (pore size, 18 nm), equipped with a Discovery C18 column, 20 × 4 mm inner diameter security guard cartridge (Supelco Inc., Bellefonte, USA). A 20-µl sample loop was employed for injection. Purified water with the addition of 0.1% formic acid was used as eluent A and acetonitrile as eluent B. The linear gradient profile was programmed from 90:10 (A%:B%, v/v) linear gradient to 76:24 in the first 10 min, increased to 40:60 in the next 2 min and changed to 90:10 in the next 3 min, and finally, the column was reconditioned for 5 min to the start conditions. Analyses were performed at ambient temperature at a flow rate of 1.0 ml/min, which was split 4:1 after the analytical column to allow 200 µL/min to enter the ESI source.

Calibration curves

Linearity of the proposed analytical method was verified by analyzing standard solutions in the range of 0.1 −100 mg/L for diazepam. The calibration curves were obtained with a determination coefficient R^2 of 0.9996 and 0.9998, respectively. The repeatability of triplicate subsequent injections was ranging from 98.5% to 99.5%, depending on the sample concentration and type of analyte.

Diazepam stability in pure water and WWTP removal efficiency

The stability of diazepam dissolved in pure water and in the activated sludge collected from the WWTP located at Birzeit University [19] was determined to ascertain if hydrolysis or biodegradation reactions were going to occur before the filtration stages. For this reason, samples were collected at specific times and analyzed by HPLC.

In the second trial the activated sludge was spiked with the same quantity of the drug and aeration was permitted to preserve the bacterial growth in the mixture. The concentration of diazepam at each time interval was determined using the calibration curve and the percentage of degraded drug was calculated as the difference from the initial concentration.

Disposable cartridges SPE-C_{18} were used to pre-concentrate 10 mL of each sample by adsorption of analytes. A part of the methanolic solution eluted from SPE cartridge (20 µL) was injected into the HPLC, and analyzed using the same conditions for the determination of diazepam. Recovery tests were performed using triplicate solutions, and values ranging from 98% to 102% were obtained.

Photolysis and Photo-catalysis

Photochemical experiments were conducted using solar irradiation system. Working solutions of diazepam (10 mg/L) were prepared when used by dilution from stock solution (100 mg/L). To investigate the effect of hydrolysis, the same experiment in the dark was performed. The tests were carried out with extreme care to ensure uniform experimental conditions during the degradation kinetics. At specific time intervals, samples of 2.0 mL were taken and immediately analyzed.

Working solutions of diazepam (10 mg/L and 0.5 mg/L) were added with 200 mg/L of TiO_2. Before the analysis, the solutions were filtered using 0.2 µm micropore cellulose acetate membrane filters (Cat. No.10462200, Schleicher and Schuell, Dassel, Germany). Experiments were carried out in triplicate and were stopped after the half-life was apparently achieved.

Rate constant measurements

Kinetic parameters (reaction order (n), determination coefficient (R^2), half-life ($t_{0.5}$), kinetic constant (k) were obtained by linear regression of logarithmic concentration values determined as a function of time.

RESULTS AND DISCUSSION

Stability of diazepam in pure water and in sludge

Diazepam was found to undergo degradation both in water and sludge. Hence, removal of this pharmaceutical from wastewater body is essential. Advanced WWTP with RO as final stage was found to be highly efficient in removing this drug and its metabolites from spiked wastewater samples. Clay-micelles complex and activated carbon were identified as good low cost adsorbents for this drug with high efficiency. The results indicate that integration of clay -micelle complex is very promising in achieving complete elimination of this drug and its degradation by-products from wastewater body [9].

Although hydroxylation is identified as the main degradation of pharmaceutical compounds in human liver, this was not evident for the degradation process of diazepam in wastewater. However similar degradation products were found in wastewater, as reported in **Fig. 4** where the transformation process of diazepam (**2**) to nordiazepam (**3**) and temazepam (**4**) is shown.

Figure 5 shows the extracted ion chromatograms (XICs) of a diazepam solution after 60 days of biodegradation using LC-FTICR MS. The ions monitored are displayed in each trace and correspond to the most abundant protonated molecules, $[M+H]^+$, using a restricted window of ±0.0010 m/z unit centered around each selected ion.

Using selective extracted ion chromatograms by FTICR MS, generated with a tight mass-to-charge ratio window of ±0.0010 units around each selected protonated molecule (i.e., $[M+H]^+$ ± 1.0 mDa), greatly reduced the signal complexity of the total ion current trace (data not shown) allowing the complete characterization of all degradation products.

For the LC-MS data analysis of diazepam peak at m/z 285.0800, degradation showed only two degradation products that were found in diazepam metabolic pathways in human (via cytochrome P450s). These metabolites are nordiazepam

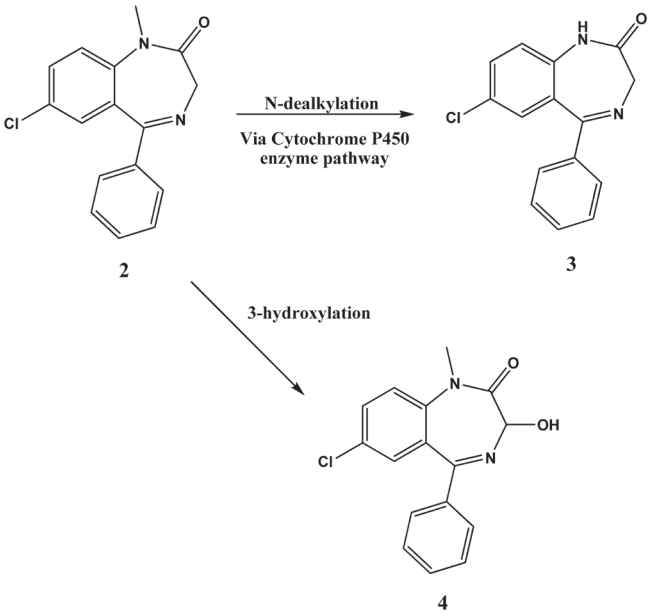

Fig. 4 Metabolism of (**2**) diazepam to (**3**) nordiazepam and (**4**) temazepam in wastewater.

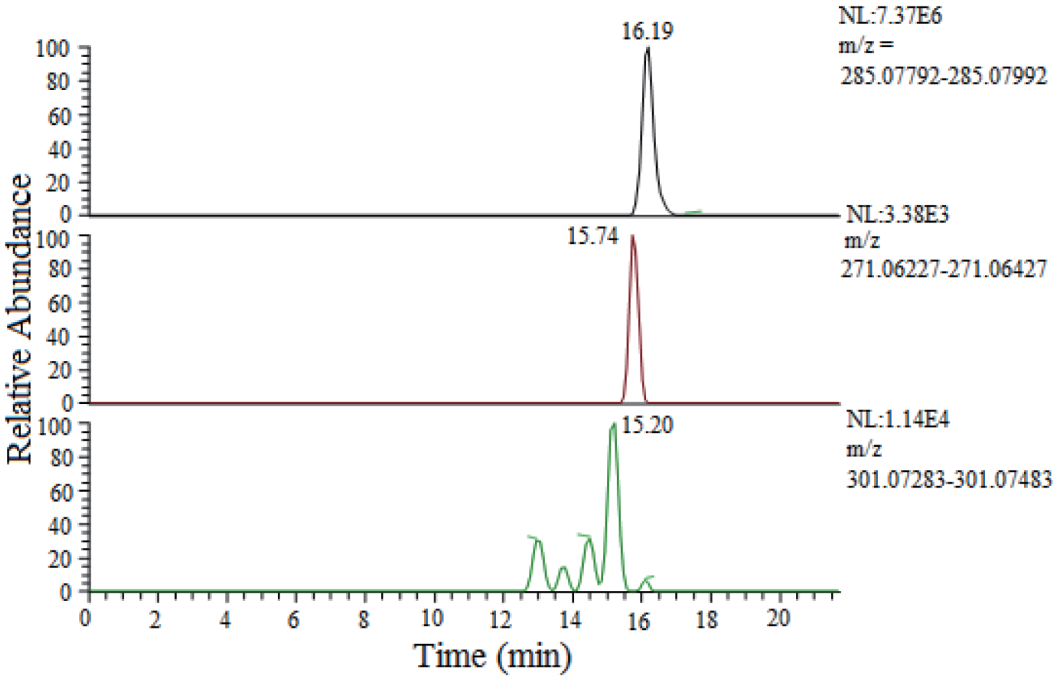

Fig. 5 Extracted ion chromatograms (XICs) by LC-FTICR MS acquired in positive ion mode of a diazepam solution after 60 days of biodegradation. The ions monitored are displayed in each trace and correspond to the most abundant protonated molecules, $[M+H]^+$, using a restricted window of ±0.0010 m/z unit centered around each selected ion.

that showed peak at m/z 271.0633 (**3**) and temazepam at m/z 301.0738 (**4**) (**Fig. 6**). Mass spectra for these compounds are reported in **Fig. 6**.

Interestingly, accurate mass data of the degradation products (metabolites), as protonated molecules, with a mass error lower than 1.7 ppm was found, indicating a good mass accuracy. Temazepam was found in different isomeric forms in respect to the position of hydroxyl group, where the most dominant was the para position [22].

To the best of our knowledge biodegradation of diazepam in wastewater was never attempted. This degradation process is similar to that of diazepam metabolic pathway in human liver mediated by cytochrome P450s. Cytochromes represent a huge family of enzymes that is found to catalyze the oxidation of a wide variety of chemicals, including many environmental pollutants such as pharmaceuticals. These enzymes are found in almost all living organisms. It is known that the P450 cytochromes can catalyze hydroxylation or dealkylation reactions of organic molecules and convert toxic chemicals into less toxic intermediates (usually more hydrophilic forms) that are more easily excreted from the body. In bacteria, P450s are involved in catabolic reactions of pharmaceuticals and are important in cleaning contamination sites. It was found that *Pseudomonas* sp. and *Bacillus* sp. frequently observed in activated sludge can produce the enzymes called cytochrome $P450_{cam}$ and $P450_{BM-3}$, respectively [8]. Therefore, P450s are likely to catalyze metabolism of diazepam. The removal of pharmaceuticals by bacteria catalyzes their metabolisms and is of great importance because municipal wastewaters from major urban areas are considered to emit these substances into the aquatic environment on a continuous basis, where they can persist for days [8].

Photolysis and photo- catalysis process

Table 1 shows kinetic parameters of the photo-chemical reactions, calculated considering three replicates for each experiment. In all cases considered in corresponding experiments, photo-reactions of the first order resulted, and the half-life of diazepam under light irradiation was always lower than diazepam under light and presence of TiO_2 .The pure standard solutions used as a control in the darkness did not show any significant degradation during the experiment.

Figure 7 shows the fraction $[1-(C_0-C_t)C_0]$ of diazepam remaining in water solution under Suntest irradiation and in the dark. **Figure 8** shows the fraction of diazepam remaining

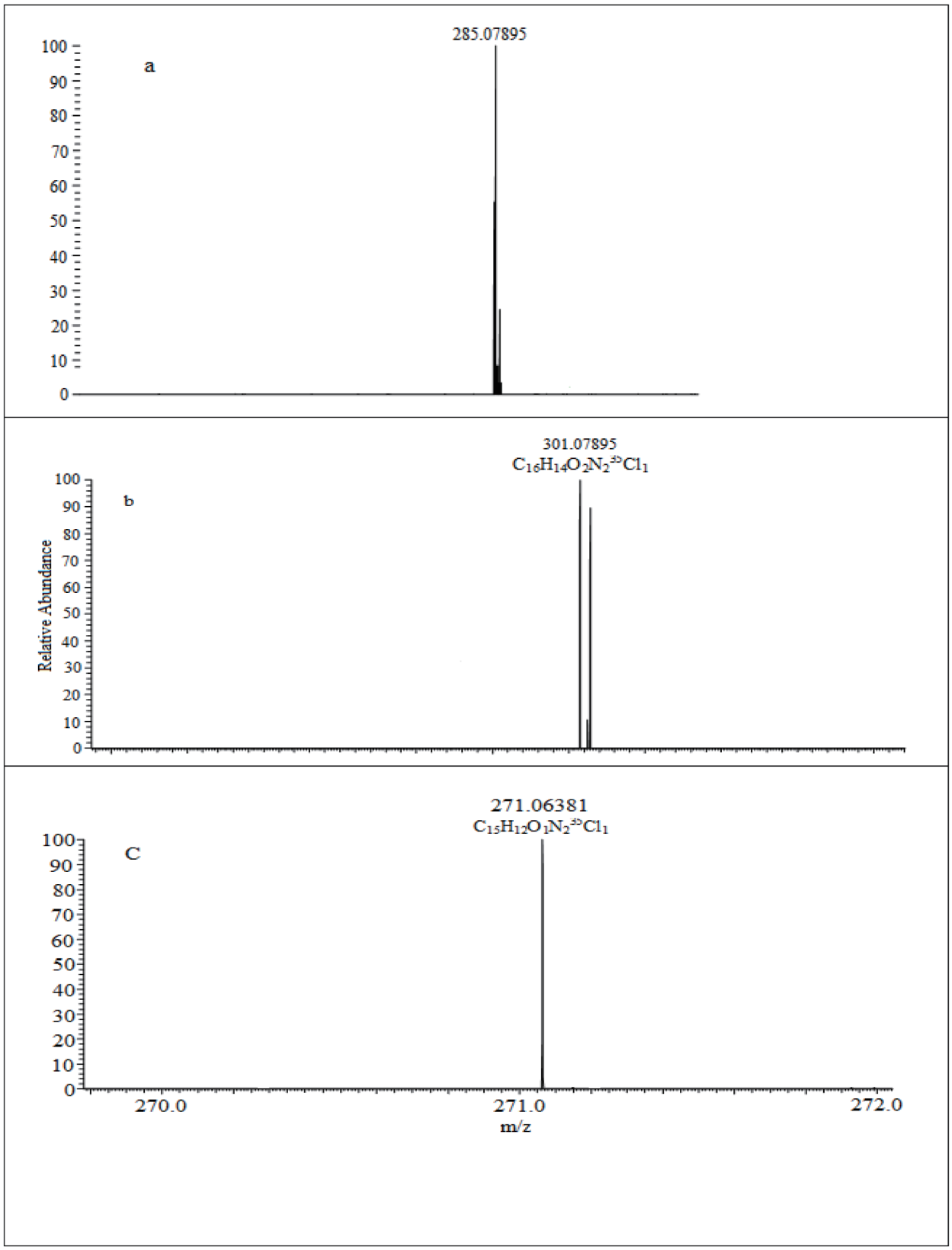

Fig. 6 Mass spectrum in positive ion mode of peak eluted (a) at 16.19 min, and simulated spectra for diazepam $[M+H]^+$ and $[M+Na]^+$ adducts, (b) at 15.84 min, corresponding to $[M+H]^+$temazepam adducts at exact m/z 301.074 and (c) at 15.74 min, corresponding to $[M+H]^+$ ion of nordiazepam adducts at exact m/z 271.063.

Table 1 Kinetic parameters of diazepam degradation: n, reaction order; $t_{1/2}$, half-life; k, kinetic constant; R^2, determination coefficient. Values were obtained on the basis of three replicate experiments.

Oxidation Process	n	$t_{1/2}$ (h)	k(h^{-1})	R^2
UV	1	34.14	0.0203	0.99
TiO_2/UV	1	5,33	0.132	0.97

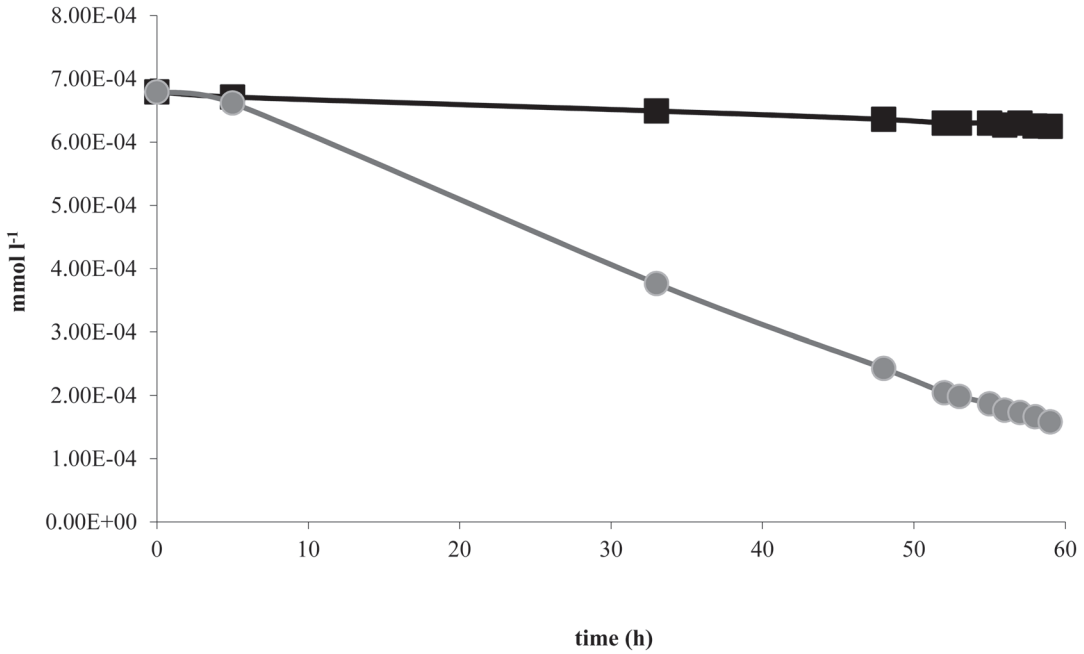

Fig. 7 Fraction of diazepam remaining in water in the dark (■) and during Suntest irradiation (●).

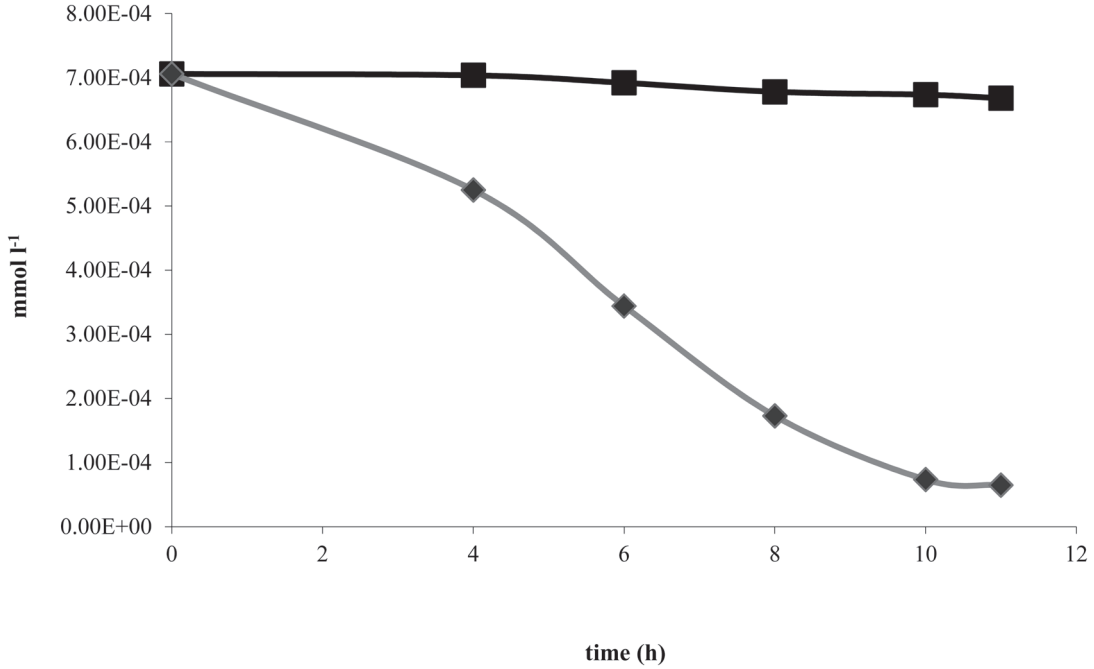

Fig. 8 Fraction of diazepam remaining in water in the dark (■) and during Suntest irradiation + TiO_2 (♦).

under Suntest in presence of TiO_2 and in the dark. the fraction $(C_0 - C_t)C_0$ is that disappeared at time *t*. Photodegradation of diazepam was much faster under light irradiation + TiO_2 (half-life = 6 hours) than under Suntest irradiation (half-life = 34 hours).

West and Rowland (2012) [18] has obtained similar results but used humic acid as photo-sensitizer (value of 103.4 and 27.6 hours for diazepam photodegradation in pure water and with humic acids respectively).

Photoproducts

It is known that TiO_2 is a strong oxidizing agent. The band gap illumination of a semiconductor particle suspended in water causes electronic transitions from the valence band to the conduction band, leaving holes in the former. These electrons and holes then either migrate to the particle surface and become involved in redox reactions or they recombine and simply liberate heat. Conduction band electrons are consumed in reactions that reduce oxidants while holes are filled via oxidation reactions. Hydroxyl radicals are generated by the oxidation of water at the valence band of TiO_2 [22–25]. All photoproducts derived from both experiments were identified either by comparison of the mass spectra and retention times with literature spectra and by accurate masses.

To identify the degradation products, the experiments were carried out by using high concentration of active product (i.e., 0.5 mg/L) that is usually not found recently in the real environment.

The main metabolites identified in the acetonitrile solutions during the first 10 h of Suntest irradiation were: (nordiazepam) (**3**), common to triasulphuron andthifensulphuron-methyl, 7-chloro-1-methyl-5-phenyl-1H-benzo[e][1,4]diazepin-2-ol (**7**), arising from triasulphuron degradation, and temazepam (**4**), occurring from thifensulphuron-methyl. After 10 h of irradiation, the formation of 4-methoxy-6-methyl-1,3,5-triazin-2-amine (**2**) (from triasulphuron and thifensulphuron-methyl), (2-chloroethoxy) benzene (**8**) (from triasulphuron), thiophene-3-carboxylic acid methyl ester (**5**) (from thifensulphuron-methyl), and a multitude of small by-products, which could not be identified with simple analytical methods, were also observed. In some samples collected after 34 h of irradiation, it was also possible to identify the acid forms of compounds '(**4**)' and '(**5**)', arising from an O-demethylation of the methoxy group.

Figure 9 depicts the proposed pathways for the formation of compounds under photolysis and photo-catalysis while **Table 2** reports the name of photo- products obtained and identified.

CONCLUSIONS

Diazepam was found to undergo degradation both in water and sludge. The degradation by products identified were nordiazepam and temazepam. Results indicate that integration of clay-micelle complex and AOPs are very promising in achieving complete elimination of this drug and its degradation by products from wastewater body.

AOPs degrade this compound to yield7-chloro-1-methyl-5-p-methoxy phenyl-1H-benzo[α][1,4]diazepin-2 (3H)-one $C_{17}H_{16}O_2N_2Cl$ (**5**); (5-chloro-2-(methyleneamino) phenyl) (phenyl) methanone $C_{14}H_{10}NOCl$ (**6**); 7-chloro-1-methyl-5-phenyl-1H-benzo[e][1,4]diazepin-2-ol $C_{16}H_{16}ON_2Cl$ (**7**); ortho, paramethoxy Diazepam $C_{17}H_{16}O_2N_2Cl$ (**8**); 5-chloro-2-methylamino-benzophenone $C_{14}H_{13}ONCl$ (**9**); 1-methyl-5-phenyl-2-hydroxy-3-hydro-1H-benzo[α][1,4]diazepine $C_{16}H_{15}ON_2$ (**10**) and 5-(4-methoxyphenyl)-1-methyl-2,3-dihydro-1H-benzo[α][1,4]diazepin-2-ol $C_{17}H_{18}O_2N_2Cl$ (**11**).

Depending on the properties of the waste stream to be treated and the treatment objective itself, AOPs can be employed either alone or coupled with other physicochemical and biological processes. Process coupling is conceptually beneficial and usually leading to improved treatment efficiency. For instance, AOPs may be employed as a pre-treatment stage initially to convert bio- recalcitrant compounds to more readily biodegradable intermediates followed by biological post-treatment. On the other hand, for effluents containing biodegradable fractions, biological pre-treatment followed by chemical post treatment maybe favorable since biodegradable compounds can be easily removed and subsequently do not compete for the chemical oxidant.

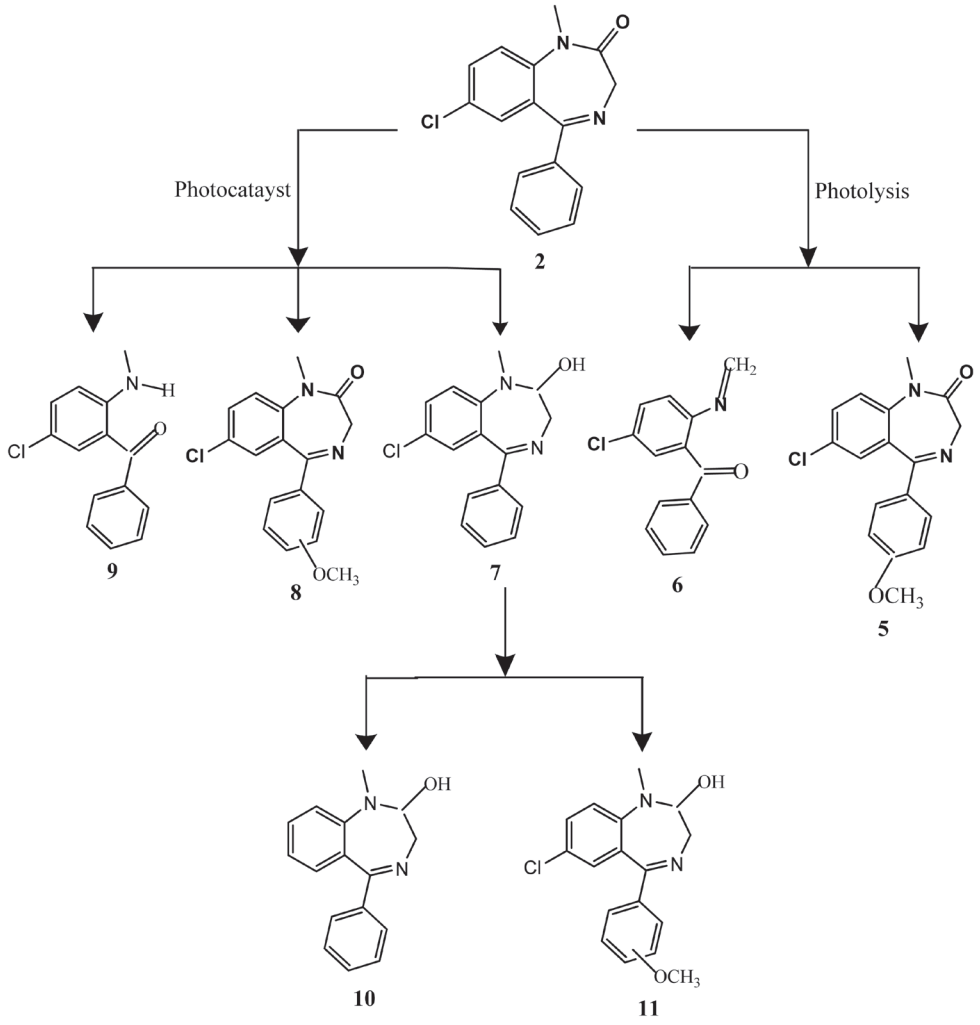

Fig. 9 Proposed pathways for the formation of compounds under photolysis and photo-catalysis.

Table 2 Photo products and photo- catalytic products of diazepam.

	Compound	Molecular formula	Accurate m/z $[M+H]^+$	Error (ppm)
2	diazepam	$C_{16}H_{14}ON_2Cl$	285.080	+0.64
5	7-chloro-1-methyl-5-p-methoxy phenyl-1H-benzo[e][1,4]diazepin-2 (3H)-one	$C_{17}H_{16}O_2N_2Cl$	315.090	+0.56
6	2-benzoyl-4-chloro-1-phenyl imine methyl	$C_{14}H_{10}NOCl$	244.050	−0.0009
7	7-chloro-1-methyl-5-phenyl-1H-benzo[e][1,4] diazepin-2-ol	$C_{16}H_{16}ON_2Cl$	287.100	+0.66
8	ortho, paramethoxydiazepam	$C_{17}H_{16}O_2N_2Cl$	315.090	−0.30
9	5-chloro-2-methylamino-benzophenone	$C_{14}H_{13}ONCl$	246.070	+ 0.57
10	1-methyl-5-phenyl-2-hydroxy-3-hydro-1H-benzo[e][1,4]diazepine	$C_{16}H_{15}ON_2$	251.120	+ 0.18
11	5-(4-methoxyphenyl)-1-methyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-2-ol	$C_{17}H_{18}O_2N_2Cl$	317.105	+ 0.19

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