Application of time-of-flight mass spectrometry to the analysis of phototransformation products of diclofenac in water under natural sunlight


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Exact mass capabilities of time-of-flight (TOF) mass spectrometry along with other mass spectrometric techniques have been evaluated to elucidate a complete range of dichlofenac phototransformation products. Photolysis experiments with diclofenac in water under direct solar irradiation were performed to characterise the main phototransformation products generated and to determine their stability. Photolysis experiments were performed in both demineralised water and reconstructed standard freshwater. Samples were extracted before analysis by solid phase extraction (SPE) with Oasis® HLB and MAX cartridges. Separation and identification of the transformation products were accomplished by the combined use of gas chromatography-mass spectrometry (GC/MS) and liquid chromatography coupled with time-of-flight mass spectrometry (LC/TOFMS). Both techniques provided complementary information that enabled the identification of 13 phototransformation products. Six of them were identified by GC/MS through the structural information provided by the full scan mass spectra obtained under electron impact (EI) ionisation and the confirmation of the molecular mass provided by positive chemical ionisation (PCI) analyses. Accurate mass measurements obtained by LC/TOFMS provided the elucidation of seven polar transformation products. The low mass error observed (<2 ppm) enabled the assignment of highly probable empirical formulas as well as identification of a process dimerisation route. The photoproducts identified demonstrated that photolysis of diclofenac occurs by two main routes. One is the consequence of the initial photocyclisation of diclofenac into carbazole derivatives. The other route goes through the initial decarboxilation of diclofenac and further oxidation of the alkyl-chain, which are typical photolytic process reactions. The main photoproduct identified was 8-chloro-9H-carbazole-1yl-acetic acid. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: diclofenac; pharmaceuticals; phototransformation; LC-TOFMS; GC-MS

INTRODUCTION

Emerging, new environmental contaminants, many of which have been unknown until recently, are the focus of much research work on their occurrence, fate and concentration levels in different environmental compartments. Among them, pharmaceuticals represent a group of increasing interest because of the high consumption derived from their use in human and veterinary medicine. As a consequence of their widespread use, a broad range of pharmaceuticals may penetrate the aquatic environment through industrial, domestic, and hospital wastewater1–3. Recent studies have demonstrated that many drugs persist through the wastewater treatment processes and are present in effluents of treatment plants, rivers and lakes4–5.

These compounds have posed an important challenge for environmental analysis. Furthermore, there is great concern about their transformation/degradation products, which can be more toxic and dangerous than the initial pollutants. Photodegradation by sunlight can be considered one of the most important processes responsible for the degradation of pollutants in environmental systems4–6–8 and understanding their degradation pathways is essential to predicting the fate and real environmental impact of these contaminants in natural waters.

However, although a variety of studies regarding the photochemical behaviour of pharmaceuticals under environmental conditions are being published, the difficulties in identifying suspected/unknown transformation products remains. Consequently, very few studies report enough consistent identification of photoproducts when classical library searches are not applicable or give low fit values. In any

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case, the identification of unknowns in complex samples requires the application of analytical tools capable of providing the complementary information necessary to complete the puzzle, leading to the final structural assignment for the wide range of compounds expected in such studies. Gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry (GC/MS and LC/MS) techniques, because of their selectivity and sensitivity, are the most powerful methods for solving the problem simply and rapidly, even when a definitive assignment of chemical structures is not possible and, therefore, only tentative degradation pathways can be proposed.

GC/MS is by far the most frequently used tool of analysis for identifying transformation products. Two important advantages of GC/MS methods are the large amount of structural information they yield for the full scan mass spectra obtained under electron impact (EI) ionisation and the possibility of using commercial libraries, making identification of unknowns feasible. However, GC/MS has important drawbacks because of its scant capability for analysing the very polar, less volatile compounds typically generated by these photo-processes. In this sense, LC/MS is a technique suitable for analytes with a wider range of polarities, and has been shown to be a powerful tool for the identification of metabolites and unknown compounds in environmental samples. But most of the analysers used provide poor fragment information, making clear assignment of the chemical structures difficult. LC/MS techniques, electrospray ionisation-time-of-flight mass spectrometry (ESI/TOFMS) does not have these disadvantages because its exact mass measurements, even when very few ions are produced by ESI, endow it with a great potential for identification.

This work studies phototransformation of diclofenac (DCFNC), a non-steroidal anti-inflammatory drug (NSAID) commonly used as an analgesic, antiarthritic and antiarheumatic. The presence of diclofenac has been reported in natural waters and in wastewater treatment plant effluents as a consequence of its incomplete elimination with conventional wastewater treatments. Direct photolysis has been described as the main transformation pathway for its elimination under environmental conditions and some photoproducts have been identified as responsible for its phototoxicity. To obtain complete information on the phototransformation pathway of DCFNC, GC/MS and LC/TOFMS techniques were employed for the identification of photoproducts generated by exposure to sunlight.

**EXPERIMENTAL**

**Standards and reagents**

Diclofenac sodium salt was purchased from Sigma-Aldrich and used as obtained. Reconstructed standard freshwater was prepared by addition of 96 mg L\(^{-1}\) NaHCO\(_3\), 60 mg L\(^{-1}\) CaSO\(_4\)•2H\(_2\)O, 60 mg L\(^{-1}\) MgSO\(_4\) and 4 mg L\(^{-1}\) KCl to distilled water, which resembles the composition of a moderately hard freshwater (American Public Health Organisation, 1998). All reagents were analytical grade.

**Hydrolysis and photolysis experiments**

Hydrolysis and photolysis experiments were performed in three-litre Pyrex beakers (UV transmissivity >80% between 320 and 400 nm, around 40% at 300 nm, internal diameter 15 cm) covered with a Pyrex top (not airtight) to avoid sample contamination and/or evaporation, and maintained under continuous stirring. During hydrolysis tests, the beakers were kept in the dark. The maximum temperature inside the beakers was 35°C.

**Sample preparation**

Solid-phase extraction was employed for preconcentration of samples prior to analysis. Two different sorbents were used to obtain wider information from the samples: Oasis\textsuperscript{®} HLB (hydrophilic lipophilic balance), which assures good recovery of compounds in a wide range of polarities, and Oasis\textsuperscript{®} MAX, a mixed-mode polymeric with reverse-phase and anion-exchange functionalities, which provides high selectivity for acidic compounds. In both cases, 50-ml aliquots were extracted from the samples and preconcentrated to 1 ml according to the following procedure: The cartridges were placed in a vacuum cube (provided by Supelco) and conditioned with 2 ml of methanol, 2 ml of deionised water, 2 ml of chlorhydric acid 0.1 N and 2 ml of water. After the conditioning step, 50-ml aliquots of the water samples were charged at a flow rate of approximately 10 ml min\(^{-1}\). Elution was performed with 2 \times 4 ml of methanol (Oasis HLB\textsuperscript{®}) or 3 ml of methanol (Oasis MAX\textsuperscript{®}) at 1 ml min\(^{-1}\). The eluates obtained were concentrated to dryness by solvent evaporation with a gentle nitrogen stream and reconstituted to a final volume of 1 ml in methanol. The extracts were stored in amber vials and refrigerated until chromatographic analysis, to prevent further degradation.

**Analytical determination**

**GC-MS**

GC/MS analyses were run on an HP 6890 Series gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) interfaced to an HP 5973 mass selective detector. Analytes were separated in an HP-5 MS capillary column (5% diphenyl 95% dimethylsioxane), 30 m, 0.25-mm i.d. and 0.25-µm-thick film. A split-splitless injector was used under the following conditions: injection volume 10 µl; injector temperature 250°C; initial pulse pressure 30 psi (1.5 min); split flow 50.0 ml min\(^{-1}\) and split time 1.5 min. The helium carrier gas flow was maintained at 1 ml min\(^{-1}\). The oven temperature programme was 1.0 min at 105°C, 25°C/min to 180°C, 5°C/min to 230°C (1 min) and the transfer-line temperature was set at 280°C. Typical MSD operating conditions were optimised by the autotuning software. EI mass spectra were obtained at 70 eV electron energy and monitored from 50 to 400 m/z. The ion-source and quadrupole analyser temperatures were set at 230°C and 106°C respectively.

Using methane as the reagent gas, analyses were also performed in positive chemical ionisation (PCI) mode. Quadrupole and ion-source temperatures were set at 120°C and 250°C respectively.
LC/TOF/MS
Liquid chromatography/electrospray/time-of-flight mass spectrometry (LC/ESI/TOF/MS), in both positive and negative ionisation, was used to detect the DCFNC phototransformation products. Selected pesticides were separated using an HPLC system (consisting of vacuum degasser, autosampler and a binary pump) (Agilent Series 1100, Agilent Technologies, Palo Alto, CA, USA) equipped with a reverse-phase 150 mm x 4.6-mm C8 analytical column and 5-µm particle size (Zorbax Eclipse XDB-C8). Column temperature was maintained at 25 °C. A and B mobile phases were acetonitrile and water with 0.1% formic acid, respectively. A linear gradient progressed from 15% A (initial conditions) to 100% A in 30 min, after which the mobile phase composition was maintained at 100% A for 5 min. The flow rate was 0.6 ml min⁻¹, and 50 µl of the matrix-matched standards and/or sample extracts were injected. This HPLC system was connected to a TOF/MS Agilent MSD TOF (Agilent Technologies, Palo Alto, CA, USA) with an electrospray interface, using the operating parameters optimised in a previous study. Accurate mass spectra were recorded across the range from m/z 50 to 1000 using LC/TOF/MS. The data recorded was processed with the Applied Biosystems/MDM-SCIEX Analyst QS software (Frankfurt, Germany) with application-specific accurate mass additions from Agilent MSD TOF software. The mass axis was calibrated using the mixture provided by the manufacturer in the m/z 50–3200 range. A second orthogonal sprayer was used with a reference solution (i.e. 6–8 mg l⁻¹). These conditions (atmospheric pressure) were selected because they are the most representative under real environmental conditions. It was not considered pertinent to perform tests in the absence of oxygen because this would not occur under natural conditions. Water would be found without oxygen only at very great depths (or at very low concentration), and sunlight does not penetrate to this depth. The results obtained in both demineralised water and reconstructed standard freshwater indicated that DCFNC undergoes spontaneous rapid phototransformation under solar illumination. However, important differences were observed between experiments with respect to pH evolution. Photolysis in demineralised water showed a spontaneous drop in pH due to chloride release that gave rise to low (total organic carbon) TOC and DCFNC as a consequence of DCFNC precipitation, as already mentioned. Obviously, the extent of this precipitation is also related to the concentration of DCFNC present. To avoid DCFNC precipitation, small quantities of sodium hydroxide were continuously added, maintaining the pH in the 7–8 range. This was unnecessary in the case of the experiment performed with standard freshwater owing to the pH buffer capacity of this type of water which kept the pH at 7.0–7.4 throughout analysis. Despite these differences, DCFNC degradation results with demineralised and standard freshwater were similar.

Figure 2 shows DCFNC concentration over time during the standard freshwater experiment. An in-depth kinetic study of diclofenac was not within the scope of this work, because information about photodegradation rates, half-lives and quantum yields for the photolysis of diclofenac has been reported elsewhere. The experiment was performed from February 12 to March 15. Vessels were exposed to the sunlight from 8:30 a.m. to 3:30 p.m. and samples were collected every two days. Time in the figure represents the normalised illumination time.
TOF-MS analysis of phototransformation of diclofenac in water

Figure 1. UV spectra between 200 and 400 nm of diclofenac (at pH 3 and 10) and Almería sunlight.

Figure 2. Kinetic of the disappearance of diclofenac by photolysis and TOC evolution during the experiment.

(UV$_{\text{prom}} = 25 \text{ W m}^{-2}$) calculated as previously described. Rapid decay of more than 70% of the initial concentration of DCFNC (45.5 mg l$^{-1}$) was observed during the first 30 h of irradiation. Afterwards the phototransformation rate slowed down markedly, probably because of the accumulation of coloured reaction intermediates acting as ‘inner’ filters. In fact, the solution became red-brown during the experiments. Despite fast dissipation of DCFNC in the solution, there was no mineralisation in any case, as shown by the TOC, which remained almost constant during the analyses. This demonstrates the formation of a great number of (transformation products) TPs, which are more resistant to the photodegradation than DCFNC itself.

Identification of transformation products

Some authors have reported fast, effective phototransformation of DCFNC under different conditions, however, an in-depth study of the photolysis pathway has not been achieved until now. Many difficulties have to be overcome for the evaluation of degradation processes: (i) the formation of a large number of unpredictable compounds that complicate their separation and consequently their identification; (ii) the different physico-chemical properties of the TPs that make their determination difficult with only one extraction and/or analytical procedure; and (iii) the absence, in many cases, of commercial standards to confirm their identity. These difficulties make other complementary analytical approaches based on MS necessary to evaluate the process as completely as possible. In this study, various strategies (GC/EI/MS, GC/PCI/MS, LC/TOFMS) have been applied and their effectiveness is discussed.

An important step in these studies is the selection of the sample preparation procedure. An extract of the samples must be prepared prior to chromatographic analysis, especially by GC. On the other hand, samples must be preconcentrated to determine the TPs present at low concentrations or poorly recovered with the selected extraction technique or procedure applied.

The sample preparation procedure proposed is based on previous experience in determining degradation intermediates. The use of SPE with polymeric sorbents is simple, but the best option in our experience. Because of their...
polar nature, many of the degradation products generated would escape LLE or C18 extraction. Oasis® HLB, a wide spectrum sorbent, was selected to provide adequate recovery of compounds in a wide range of polarities. Furthermore, because of the presence of an acidic functionality in the diclofenac molecule, a sorbent with anion-exchange functionalities was also used, which provided good results in the identification of acidic intermediates. Oasis® MAX cartridges contain a mixed-mode polymeric sorbent with reverse-phase and anion-exchange functionalities, which provide high selectivity for acidic compounds. The elution procedure, as described in the experimental section, eluted compounds retained by hydrophobic interaction (neutral and basic compounds) and locked in acidic compounds (not recovered by elution). Comparison of both (HLB and MAX) SPE extracts was very useful for confirming the presence of acidic functionalities in the TPs detected.

In a first approach, extracts were analysed by GC/EI/MS. All the peaks present in the chromatograms were investigated and six of them identified as possible TPs (Fig. 3). The structures of these compounds, represented in Fig. 4, were tentatively assigned by their full scan mass spectra in agreement with previous knowledge about these photoreactions. Two of them (C-5 and C-11) were confirmed by library search. Figure 3 shows the EI mass spectra of C-5, which was identified by the Wiley275 library as 3-chlorocarbazole with an 83% fit. Spectral databases available constitute a powerful tool in the identification of unknowns by GC-MS. However, its usefulness is sometime hampered by the complexity of the samples. Co-elutions of compounds and interferences make correct identification of the target compounds difficult, especially when they have not previously been described because of the complex spectra obtained. The analyses performed by PCI using methane as the reagent gas help solve this problem. The simplicity of the PCI spectra and the presence of characteristic methane adducts at \( m/z \) \([M + 29]^+\) and \([M + 41]^+\) enabled us to detect the presence of many molecular ions in the same spectrum, thus making possible separation of the co-eluting compounds by single ion selection. In addition, PCI analysis provided accurate confirmation of TP molecular weight, essential for structural elucidation. To this end, previous knowledge about the structure of the parent compound is also relevant. For example, compounds 8 to 11 presented a fragmentation pattern at low \( m/z \) similar to DCFNC because of the biphenylamine moiety they have in common. Presence of characteristic isotopic clusters and typical \([M – Cl]^+\), \([M – CHO]^+\) and \([M – CH_3]^+\) losses enabled tentative structure assignation.

Since direct injection of the extracts into the GC system was preferred to the application of tedious and complex derivatisation procedures, highly polar or non-volatile TPs could escape the scope of the technique. So the application of LC/MS was an unavoidable and attractive option. The application of various LC/MS strategies to the identification of unknowns has already been evaluated and discussed19–24. ESI/TOF/MS was selected in this study for its capability to obtain accurate mass spectra, facilitating identification. On the basis of these sensitive mass analyses, the system’s program calculates the elemental composition and empirical formula of the protonated molecules, providing a list of hits that best match the mass measured. Chlorine

![Figure 3. Typical GC/EI/MS chromatogram obtained after three days of solar exposition where six photoproducts were identified. Example of full scan mass spectrum corresponding to compound 5(inset) (Fig. 4).](image-url)
isotopic patterns and additional information on the atomic composition can be used to limit the number of possible hits, which in all the cases studied was reduced to a single option. With this procedure, seven polar PTs were identified. Figure 5 shows an LC/TOFMS chromatogram and an example of the elemental composition estimation used to identify compound 13 (8-chlorocarbazole-1-formic acid), where data relative to the experimental and calculated masses of the deprotonated ion, the error between both masses and the proposed empirical formula corresponding to the identified compounds are shown. Data corresponding to the rest of photoproducts identified are included in Table 1. Results for the accurate mass of these compounds were found at an error less than 0.3 mDa or 1.0 ppm in all cases.

The tentative structure assigned to these compounds is shown in Fig. 4. Accurate mass measurement of fragment ions in the MS spectra also became particularly important in the structure elucidation of the TPs. Carboxylic acid functionalities (compounds C-1, C-6, C-7 and C-13) were confirmed by presence of the [M – H – COO]⁻ fragment in the ESI(−) spectrum (Fig. 5). The ions at m/z 258, 240, 254 and 246 all show a loss of 43.9898 amu ±0.0002, which is the correct mass loss for CO2 (calculated loss of CO2 is 43.9898 amu). This demonstrates the loss of CO2 and indicates the presence of the carbonyl group in the structure of the degradate. The protonated molecule of diclofenac undergoes the same loss of CO2 and is the key to the understanding the phototransformation products. The accurate mass and empirical formula also obtained from the fragment ions assured correct identification of these compounds. Furthermore, these compounds were not present in the Oasis® MAX SPE extracts with the elution procedure applied because of their strong retention in the anion-exchange functionalities. Compounds C-3 and C-12 were characterised by the appearance in their ESI(+) spectra of the [M – H2O]⁺ fragment, also confirmed by accurate mass measures, typical of alcohols. Although it is not possible to predict the exact position of the OH group in the molecule, a tentative structure has been proposed in Fig. 4.

Along with the photoproducts already mentioned, the presence of dimer products could also be detected during the photolysis of DCFNC. Dimer formation has previously been reported in photodegradation processes25. These compounds have not been assigned, but considering some characteristics of their structure, such as the presence of acidic groups or absence of chlorine atoms, it can be hypothesised that they are formed from high mass DCFNC photoproducts coexisting in the reaction mixture. Analyses recorded with a UV detector at 400 nm have revealed that some of these dimer products (MW 494, 481 and 524) are the most abundant coloured compounds at this wavelength, being partly responsible for the colour developed during the phototransformation process. One example is the ion at accurate mass m/z 479.1248, which gives the best fit formula of C28H20N2O6 (neutral molecule). This formula is exactly twice that of C-6, which suggests dimer formation. The ion at m/z 493.1041 is also closely related to C-6, differing by only 1 oxygen and 3 hydrogen atoms. Thus, it appears that losses from chlorine form the carbazole structure followed by hydroxyl substitution of chlorine to form C-6. This structure

Figure 4. Scheme of the proposed phototransformation pathway for diclofenac under sunlight exposition.
Figure 5. LC/TOFMS chromatogram obtained in negative ion mode. Example of elemental composition calculation and ESI/mass spectrum used for the identification of compound 13 (8-chlorocarbazole-1-formic acid).

**Table 1.** Accurate mass measurements obtained from the LC-TOFMS spectra of the DCFNC (de)protonated phototransformation products identified.

<table>
<thead>
<tr>
<th>Compound (ionization mode)</th>
<th>Formula</th>
<th>Calculated mass (m/z)</th>
<th>Experimental mass (m/z)</th>
<th>mDa error</th>
<th>ppm error</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1 (ESI−)</td>
<td>C_{14}H_{9}NO_{2}Cl</td>
<td>258.0327</td>
<td>258.0330</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>C-3 (ESI+)</td>
<td>C_{13}H_{11}NOCl</td>
<td>232.0523</td>
<td>232.0524</td>
<td>&lt;0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>C-4 (ESI+)</td>
<td>C_{13}H_{10}NOCl</td>
<td>230.0367</td>
<td>230.0368</td>
<td>&lt;0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>C-6 (ESI−)</td>
<td>C_{14}H_{9}NO_{2}</td>
<td>240.0666</td>
<td>240.0667</td>
<td>&lt;0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>C-7 (ESI−)</td>
<td>C_{14}H_{9}NO_{2}Cl</td>
<td>254.0458</td>
<td>254.0460</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>C-12 (ESI+)</td>
<td>C_{13}H_{10}NO_{2}Cl</td>
<td>282.0083</td>
<td>282.0084</td>
<td>&lt;0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>C-13 (ESI−)</td>
<td>C_{13}H_{10}NO_{2}Cl</td>
<td>246.0327</td>
<td>246.0329</td>
<td>0.2</td>
<td>0.7</td>
</tr>
</tbody>
</table>

is activated by the phenol structure and is easily dimerised. This pathway leads to all chlorine-free dimers. This is a major pathway turning the solution brown.

**Phototransformation pathway**

In the light of the PTs detected, a tentative phototransformation pathway has been proposed as shown in Fig. 4. Only qualitative and semi-quantitative information has been obtained. A kinetic study was not possible for the majority of the photodegradation products identified, since the concentration of these compounds is unknown due to the lack of analytical standards. The most significant DCFNC phototransformation process is the photocyclisation to the corresponding monohalogenated carbazole (C-1) (route I). This process has already been reported elsewhere\(^\text{17,18,26}\) and is the starting point of other consecutive processes mainly affecting the alkyl-chain. It was found that dehalogenation of the chlorocarbazole moiety plays only a minor role in phototransformation, the chlorine atom being present in most of the photoproducts detected (I\(_b\)). However, the substitution of the chlorine atom in the hydroxyl-group has been observed with the formation of 8-hydroxy-9,9a-dihydro-4aH-carbazol-1-yl) acetic acid (C-6) (route I\(_b\)). As previously explained, this highly reactive product is the precursor of dimer formation. The formation of carbazoles is of great interest because of its relationship to the phototoxicity caused by DCFNC\(^\text{17}\).

In addition to this phototransformation route, an alternative pathway was also observed (route II). This route starts with decarboxylation of DCFNC (C-8) and further oxidation of the alkyl-chain (C-9 to C-13). Presence of this alternative route has not previously been reported in previous DCFNC
photolysis studies and represents an important contribution to the transformation of this compound. Compounds C-8 to C-10 and C-11 were identified by GC/MS, C-10 being the most abundant. All of them turned out to be very stable, remaining until the end of the experiment. In LC/MS, C-13 also showed an intense and very stable peak. Compounds with lower molecular weight corresponding to break-up of the carbazole or biphenylamine structure were not identified suggesting the stability of these structures.

CONCLUSIONS

In this study, we have shown that both LC/ESI/TOFMS and GC/MS are powerful techniques for the chemical elucidation of DCFNC phototransformation products. The use of LC/ESI/TOFMS has proved to be one of the most powerful approaches currently existing to investigate suspected or unknown TPs. The information obtained on elemental composition of the by-products and their fragment ions with very low mass errors (<2 ppm) clearly constitute much faster, higher order identification than that afforded by nominal mass measurements. The methodology followed in this paper, based on the combination of SPE and GC/LC/MS can easily be applied to other families of compounds in real field studies.

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