Fluorouracil in the environment: Analysis, occurrence, degradation and transformation

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\begin{abstract}
5-Fluorouracil (5-FU) is a fluorinated pyrimidine analogue important in the treatment of cancer whose fate in the environment is yet to be fully addressed. Due to its high polarity 5-FU requires challenging sample preparation and therefore we thoroughly investigated different solid phase extraction mechanisms (ion pair, ion exchange, reversed phase), sorbents and derivatisation agents to enable trace-level analysis of 5-FU based on GC–MS/MS in natural and wastewaters. Ion pair and ion exchange retention mechanisms enable the extraction of 5-FU from deionised water, but were inappropriate for complex environmental matrices, where the reversed phase sorbent Isolute ENV+ gave the best extraction efficiencies (53% and 93% for wastewaters and surface waters, respectively). Further, alkylation was rejected in favour of silylation with MTBSTFA. The achieved limits of quantification (LOQ) for waste and surface waters were 1.6 ng/L and 0.54 ng/L, respectively. The method was used to analyse samples of hospital, wastewater treatment plant influent and effluent and surface waters. 5-FU was quantified in four out of the twelve samples of oncological ward wastewaters and municipal wastewater treatment plant influents in concentrations from 4.7 ng/L to 92 ng/L. This work is also the first to study the environmental transformation of 5-FU and its prodrug capecitabine (CAP). Their removal and transformation was simulated using a series of biodegradation and photodegradation experiments, where 5-FU proved more degradable in comparison to CAP. Transformation of 5-FU and CAP was studied by using ultra-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC–QqTOF). Overall, six transformation products for 5-FU and ten for CAP are proposed; 13 of these are to our knowledge published for the first time.
\end{abstract}

1. Introduction

Anticancer, cytostatic, cytotoxic and antineoplastic drugs are synonyms for a class of pharmaceuticals used to prevent growth and development of malignant cells and neoplasms in cancer patients [1]. The Anatomical Therapeutic Classification (ATC) scheme divides cytostatic compounds into five classes according to their mode of action: alkylating agents, antimitabolites, plant alkaloids and other natural products, cytotoxic antibiotics and related substances, and, other antineoplastic agents. 5-Fluouracil (5-FU) and its prodrug capecitabine (CAP) belong to the class of antimitabolites. CAP is a fluoropyrimidine carbamate which is rapidly metabolised to the active substance 5-FU in the body, but to a higher degree it is converted in tumour cells than in normal tissues. It works by inhibiting DNA synthesis and slowing tumour growth. CAP allows more patient-friendly oral administration, improved quality of life and economic advantage and unlike intravenously administered 5-FU, has fewer and less severe side effects such as gastrointestinal toxicity [2]. The trend is towards the prescription of CAP over 5-FU [2] with consumption of both compounds measured in tonnes annually for European countries [3–5] and while this is low compared to other classes of pharmaceuticals [3], but these drugs are highly potent and are known to have cancerogenic, genotoxic, mutagenic and teratogenic properties.

Not all the administered drug is metabolised. Typical metabolism rates for 5-FU and CAP are 0.85 and 0.70, respectively [3]. The remainder, a mixture of parent compounds and metabolites, are excreted from the body and typically enter the sewerage system eventually reaching surface waters. Little if anything is known about their eventual fate and to the best of our knowledge there exist no studies regarding their environmental transformation [2].

Kosjek and Heath [2] provide a critical overview of the available literature on sample preparation and analytical methods for determining 5-FU in waste and environmental waters. They find...
that only recently with developments in chromatography and mass spectrometry has it become possible to determine 5-FU in environmental concentrations (sub ng/L) with limit of quantification (LOQ) as low as 5–50 ng/L [6,7]. As a consequence, 5-FU has only been quantified in one study by Kovalova et al. [6], where 6 out of 10 hospital wastewaters had a maximal and median concentration of 27 ng/L and <LOQ, respectively. Other attempts to quantify the compound in wastewaters, hospital effluents and surface waters [2,8] failed due to insufficient method sensitivity. Negreira et al. [9] are the only ones to report the occurrence of CAP in municipal wastewaters in levels up to 25 ng/L.

Understanding the fate of these drugs in the environment and recognising their transformation products is important since chronic exposure to even trace amounts has the potential to cause adverse effects on non-target organisms [4]. This can only be achieved through the development of suitable analytical methods capable of determining ultra low amounts. For this reason the aims of our study were: (i) to develop an analytical method capable of determining 5-FU in environmental and wastewater samples in sub ng/L; (ii) to determine the environmental and wastewater concentrations of 5-FU residues; (iii) to evaluate the removal of 5-FU and CAP during biological and abiotic water treatment and (iv) to identify stable transformation products (TP) formed during treatment processes.

2. Materials and methods

Caution: 5-FU and CAP are cytotoxic, genotoxic, mutagenic and teratogenic, and should be handled carefully; the operator should protect himself against UV irradiation.

2.1. Standards, reagents and chemicals

5-FU (5-fluoro-1H,3H-pyrimidine-2,4-dione, CAS: 51-21-8) was of ≥99% purity and was obtained from Sigma–Aldrich (St. Louis, MO, USA). Deuterated 5-FU (5-fluorouracil-6-d1; purity: chemical 98%, isotopic 99%; CAS: 90344-84-6) was purchased from LGC Standards GmbH (Wesel, Germany). 5-Chlorouracil (5-CU, 5-chloro-1H,3H-pyrimidine-2,4-dione, CAS: 1820-81-1) was of 98% purity and was obtained from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada), while CAP (pentyl [1-(3,4-di hydroxy-5-methyltetrahydrofuran-2-yl)-5-fluoro-2-oxo-1H-pyrimidin-4-yl][carbamate] was purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). The derivatising agents tested were MTBSTFA (N-methyl-N-tert-butyldimethylsilyl) trifluoroacetamide) purchased from Acros Organics (Geel, Belgium), MSTFA (N-methy-N-(trimethylsilyl) trifluoroacetamide), BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) and PFBBr (pentafluorobenzyl bromide) obtained from Sigma–Aldrich. All solvents (ethyl acetate, toluene, hexane, pyridine, methylene chloride, acetone, acetonitrile, methanol, water) and chemicals were of analytical grade purity.

Stock solution for quantitative analysis of 5-FU in waste and surface waters was prepared in methanol in concentration of 0.2 mg/mL. Calibration standards were prepared by further dilution in methanol. To prevent radical scavenging during photodegradation and growth of biomass on methanol we prepared stock solutions of 5-FU and CAP in water and diluted them as appropriate.

2.2. Sample collection

Aqueous samples from two sets of hospital – wastewater treatment plant – receiving water (A and B) were sampled. Wastewater from Hospital A's oncological ward was collected from a neutralisation basin on two occasions in 2012, while two 24-h time proportional composite samples were collected from wastewater treatment plants (A: influent and effluent) in 2012. Two additional samples were collected: one at the effluent channel just before entering the receiving surface water and another one, a grab sample, from the receiving river 50 m downstream of the wastewater effluent outflow.

Hospital B is a specialised hospital that includes cancer therapy, but does not include 5-FU in the medical therapy. Grab samples were collected from influent and effluent of the corresponding hospital WWTP (B) as well as from stream water (grab sample) before and after the confluence point. A detailed description of the samples is given in Table 2. Samples were initially filtered through glass microfibre filters (Machery Nagel, Dueren, Germany), and then through 1.2 µm cellulose nitrate filters (Whatman, Dassel, Germany). Samples were stored at –20 °C prior to analysis. Method development involved using artificial wastewater samples obtained from bench scale bioreactors [10,11].

2.3. Sample preparation

Sample preparation depended on the type of analysis performed: GC–MS/MS or LC–MS/MS. All GC–MS/MS samples were intended for quantitative analysis of 5-FU and were prepared using solid phase extraction (WW, SW or DEG sample preparation protocol) and derivatised prior to analysis. The sample preparation protocol depended on a sample matrix and expected concentration of the analyte:

(a) “WW sample preparation protocol” was employed for determination of 5-FU in trace level concentrations in actual wastewater samples. 1 g/6 mL Isolute ENV+ cartridges (Biotage AB, Uppsala, Sweden) were conditioned with 6 mL methanol, equilibrated with 6 mL deionised water, then enriched with 100 mL wastewater samples (pH 6), vacuum-dried and eluted with 3 mL × 2 mL of methanol. The extracts were dried under nitrogen stream and dissolved in 150 µL ethyl acetate.

(b) “SW sample preparation protocol” was employed for determination of 5-FU in trace level concentrations in surface water samples. This protocol is identical to “WW sample preparation protocol”, apart from the sorbent mass which was in the latter case 500 mg of Isolute ENV+ sorbent per 6 mL cartridge.

(c) “DEG sample preparation protocol” was employed for determination of 5-FU in concentrations higher than 100 ng/L in samples from biodegradation and photodegradation experiments. 100 mg/3 mL Isolute ENV+ cartridges were conditioned with 3 mL methanol, equilibrated with 3 mL deionised water and enriched with 3 mL samples with pH set to 6 using sodium acetate buffer. As follows, the cartridges were vacuum-dried and eluted with 3 mL × 2 mL of methanol. These eluates were nitrogen-dried and reconstituted in 0.5 mL of derivatisation solvent ethyl acetate.

As follows, samples prepared by WW, SW or DEG sample preparation protocol were derivatised using 30 µL MTBSTFA at 80 °C for 1 h and analysed with GC–MS/MS.

LC–MS/MS was employed for identification of CAP and 5-FU transformation products. No sample pretreatment was necessary for samples from the phototransformation experiments, whereas those obtained from biodegradation experiments were, prior to LC–MS/MS, analysis filtered through 0.2 µm reversed cellulose filters (Phenomenex, CA, USA).

2.4. Instrumental analysis

2.4.1. GC–MS/MS

An Agilent 4500-GC hyphenated with an ion trap 240–MS mass spectrometer was employed to determine 5-FU. The GC oven was
programmed as follows: an initial temperature of 65 °C was held for 2 min, then ramped at 30 °C/min to 180 °C, ramped at 7 °C/min to 220 °C, at 40 °C/min to 300 °C, and finally held for 3 min at this temperature. The total GC run time was 16.55 min. A DB-5 MS column (30 m × 0.25 mm × 0.25 μm (Agilent J&W, CA, USA)) capillary column was used, with He as the carrier gas (37 cm s⁻¹). One-microlitre samples were injected at 250 °C in splitless mode, kept until 3 min, and then split at 1/30. The ion trap MS was operated in electron impact (EI) ionisation mode with an external ionisation source at 15 μAmps emission current and a target ion count of 20,000. All MS/MS transitions were performed in a resonant waveform type at following excitation amplitudes: 1.19 V for 5-FU, 1.20 V for 5-FU-d1 and 1.24 V for 5-CU.

To confirm the identity of 5-FU in actual water samples we applied the following criteria:

- Retention time match: tR (5-FU) = 9.7 min ± 0.1 min;
- MSMS spectrum match: NR > 700;
- Product ion ratio 301 > 187 (qualitative m/zj) to 301 > 167 (qualitative m/zj): peak area (m/zj 187)/peak area (m/zj 167) ≤ average ratio in quality assurance results ± 20%.

MS Workstation v6.9.3 software was used for control, automation and processing.

2.4.2. LC–MS/MS

To follow the degradation pattern of CAP and 5-FU, and to identify their transformation products a Waters Acquity Ultra-performance liquid chromatograph (UPLC, Waters Corp., Milford, MA, USA) coupled to a Waters Premier hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (QqToF) was employed. The chromatographic separation was performed on the UPLC equipped with a binary solvent delivery system and an autosampler. The injection volume was 7.5 μL. Separation was achieved using a 5-cm-long Acquity UPLC™ BEH Shield C-18 (Waters Corp.) column with 1.7 μm particle size and 2.1 mm internal diameter. Compounds were analysed under positive (ESI+) and negative (ESI−) ionisation conditions. The mobile phases used in ESI(+) were (A) 0.01% formic acid and (B) acetonitrile. To enable the separation of CAP transformation products the LC separation in ESI(+) was adopted to a relatively long elution gradient, where % B was increased from 10% to 40% in 7 min, afterwards it was again decreased back to 10% in 0.5 min and was kept isocratic for 1.5 min. In total, the run took 9 min. In case of ESI(−) analysis 0.01% formic acid was swapped with LC–MS grade water and the elution gradient was as follows: 100% A for 0.8 min, then the % of B was increased to 90% in 4.2 min, then decreased back to 0% B in 0.5 min and kept isocratic for 1.5 min. Flow rate was 0.3 mL min⁻¹ in both separation methods and the column temperature was maintained at 50 °C in ESI(+), and at 30 °C in ESI(−) analysis. The UPLC system was coupled to QqToF equipped with an electrospray ionisation interface. The capillary voltage was set to 3.0 kV in ESI(+) and to 2.8 kV in ESI(−), while the sampling cone voltage was 20 V. Source and desolvation temperatures were set to 130 and 300 °C, respectively. The nitrogen desolvation gas flow rate was 530 Lh⁻¹. For MS experiments, the first quadrupole was operated in rf-only mode, while detection was performed in the ToF mass analyser. MS data were acquired over an m/z range of 50–1000 at collision energy of 4 V. For MS/MS operation, the acquisition range was between m/z 50 and 400, and argon was used as the collision gas at a pressure of 4.5 × 10⁻³ mbar in the T-wave collision cell. The MS/MS experiments were performed with collision energy, varied between 10 and 40 V, to generate product ion spectra providing the most structural information. Data were collected in centroid mode, with a scan accumulation time set to 0.25 s and an interscan delay of 0.02 s. The data station operating software was MassLynx v4.1. Prior to analysis, the instrument was calibrated over a mass range 50–1000, using a sodium formate calibration solution. Reproducible and accurate mass measurements at a mass resolution of 10 000 were obtained using an electrospray dual spray with [M+H]⁺ 554.2615, [M−H]⁻ 556.2271 as the reference compound. The latter was introduced into the mass spectrometer alternating with the sample via a Waters Lock Spray device. Elemental composition of transformation products was calculated from accurate masses determined by high resolution mass spectrometry (HRMS).

2.5. Validation of analytical method for determination of 5-FU

The method performance was evaluated by estimating linearity, repeatability, accuracy and sensitivity, SPE efficiency and matrix effects. The linearity of the method was determined by plotting the peak area ratio of 5-FU to its deuterated analogue versus drug concentration. The r² value was determined from the regression curve. Repeatability was estimated as instrumental (repeatability of measurement) and method repeatability (repeatability of sample preparation and analysis). The instrumental repeatability was determined as standard deviation of three consecutive injections of the same sample. The method repeatability was determined as standard deviation obtained by analysis of 5-FU in three replicate samples. The accuracy error was expressed by [(mean observed concentration – spiked concentration)/spiked concentration] × 100%. Sensitivity was expressed as limit of detection (LOD) and limit of quantification (LOQ). LOD was determined as 3-times the standard deviation of the base line for three blank samples of artificial wastewater or surface water. LOQ was calculated as 3.33-times the LOD. SPE efficiency was determined as a quotient of mean peak area of 5-FU spiked before SPE with that obtained by spiking the same amount of 5-FU into the extract post SPE. Matrix effect was determined by comparing slopes of the calibration curves prepared in artificial wastewater and in tap water.

2.6. Setup of biodegradation experiments

Biodegradability of 5-FU and CAP was tested by conducting batch biodegradation experiments employing three concentrations of activated sludge (AS, 0.67 g/L, 0.14 g/L and 0.014 g/L). To account for possible abiotic degradation a series of control samples were run including experiments in absence of AS and or nutrient-mineral medium. Additionally, we studied the impact that the concentration of 5-FU (1 μg/L, 1 mg/L, 10 mg/L, 20 mg/L and 100 mg/L) has on its biodegradation rate. A detailed description of the experiment can be found in the Supplementary material. The composition of the nutrient-mineral medium is described in Kosjek et al. [10].

2.7. Setup of the photodegradation experiments

Photodegradation studies were performed in a cylindrical glass reactor by exposing 760 mL of aqueous solutions of 5-FU or CAP in deionised water (initial concentration 1 mg/L) to UV irradiation. Monochromatic low pressure (LP) mercury UV lamp with peak emission at 254 nm was used as the source of irradiation. The light intensity, which was measured by ferrooxalate actinometry, was 0.78 × 10⁻⁶ Einstein/s. The duration of the UV experiment and sampling frequency were adopted according to the TP identification process. In general, the duration of UV experiment was within 10 min and 24 h. 4 mL sub-samples were withdrawn during the course of the experiment. A second experiment was also performed with the addition of H₂O₂ (0.03%, v/v).
3. Results and discussion

3.1. Development and validation of the analytical method for determining 5-FU in aqueous matrices

3.1.1. Selection of the derivatisation conditions for determining 5-FU by GC–MS analysis

Three silylation reagents (MSTFA, MTBSTFA and BSTFA) and an alkylation reagent PFBBr were compared to determine which one gave the optimum chromatographic response, hydrolytic and thermal stability of 5-FU derivatives prior to the GC–MS with EI. Silylation was performed immediately prior to analysis in non-aqueous matrix, where the derivatisation temperature (40, 50, 60, 70, 80 °C), time (15 min, 30 min, 1 h, 8 h), derivatisation solvent (acetonitrile, ethyl acetate, toluene, hexane, pyridine and methylene chloride) and amount of derivatisation agent (30 and 50 μL) were optimised. Among the three silylating reagents MTBSTFA was favoured due to the best hydrolytic stability of the derivatives [12], and that it gave the most favourable fragmentation and superior chromatographic response. Using MTBSTFA the derivatisation yield increased from 40 °C to 60 °C and remained stable by further increase to 80 °C, therefore the latter was selected as the derivatisation temperature. Further, by testing different derivatisation solvents including acetonitrile, ethyl acetate, toluene, hexane, pyridine and methylene chloride it was shown that the choice of derivatisation solvent most crucially affects the derivatisation efficiency. A graph revealing how chromatographic response of MTBSTFA derivatives of 5-FU and 5-CU depends on a derivatisation solvent is shown in the Supplementary material (Fig. SM 1). The results show that ethyl acetate is the most effective derivatisation solvent.

An alkylation protocol was adopted from Tauxe-Wuersch et al. [7], where the catalyst K2CO3 (25%, w/w in analytical grade water) was added into the reaction mixture. Temperature (50, 60, 70, 80 °C), length of derivatisation (0.5 h, 1 h and overnight) and amount of the alkylation agent PFBBr were optimised. After derivatisation water was removed by Na2SO4 and an additional clean-up step with silica was performed. The optimum chromatographic response of the derivatised 5-FU and 5-CU was achieved after 1 h alkylation at 70 °C; however the reaction resulted in multiple peaks all involving the base fragment ion at m/z 181. This fragment ion is characteristic, not for the analyte itself, but for derivatisation with PFBBr, which will in turn reduce method selectivity. Therefore GC–MS with EI is not considered an appropriate method for determination of PFBBr derivatives. Instead, GC–MS with a chemical ionisation or GC coupled to an alternative detection method such as electron capture detection might enable an improved and more selective determination of PFBBr derivatives. Even so, for simplicity reasons the alkylation was discarded and MTBSTFA derivatisation (30 μL, 80 °C, 1 h, in ethyl acetate) was selected as the derivatisation method of choice.

3.1.2. Solid phase extraction (SPE)

5-FU is a weak acid (pKₐ 8) and an extremely polar compound with a log Kow of ~1, which makes it difficult to extract using reversed phase solid phase extraction. During the SPE method development, different SPE sorbents were tested, which can be classified according to their retention mechanisms: reversed phase, anion exchange and ion pairing. Initially, the most promising recoveries were achieved using the Oasis MAX sorbent (Waters Corp.), where the anionic interactions of negatively charged 5-FU at pH ≥ 10 were exploited. Yet, this sorbent was shown to be efficient for low-volume simple matrix samples only, since even a slight increase in a matrix complexity or ionic strength (e.g. switching from base to buffer to adjust pH in a deionised water sample or changing from deionised to tap water) radically reduces the efficiency of anion extraction. For this reason Oasis MAX was considered inappropriate for extraction of 5-FU from complex environmental samples.

An alternative to anion exchange SPE is an ion pair SPE (IP SPE), which is appropriate for highly-polar analytes that exhibit poor retention on reversed phase sorbents. The principle of IP is similar to anion exchange, but it employs an IP agent, which contains a nonpolar portion that is retained on a reversed phase sorbent, and a charged polar portion that interacts with a charged group on the analyte [13]. We tested two IP agents, triethylamine and tert-butyl ammonium chloride to enhance extraction of 5-FU. Addition of triethylamine into the equilibration phase or also into the sample matrix did not affect the retention of 5-FU, which may result from poor retention of triethylamine on the reversed phase sorbent. In contrast, tert-butyl ammonium chloride that contains longer nonpolar chains did improve the extraction efficiency on reversed phase sorbent at approximately 20%. Optimum retention was obtained on the Isolute ENV+ sorbent and by adding 0.05 M tert-butyl ammonium chloride into the equilibration phase, but not into the sample matrix, which was adjusted to pH 10 (detailed results are given in the Supplementary material). Unfortunately, IP extraction failed when changing to more complex matrices such as wastewater and was therefore discarded from further research. Nevertheless, the IP SPE remains a viable alternative for extracting 5-FU from simple matrices such as pharmaceutical samples.

A number of reversed phase sorbents were tested including Oasis HLB, Evolute ABN, Strata X and Isolute ENV+, where the hypercross-linked hydroxylated poly styrene divinylbenzene copolymer Isolute ENV+ (Biotage, Uppsala, Sweden) was the only efficient sorbent, but matrix complexity and extraction volumes had a negative impact on extraction efficiency. The highly polar nature of 5-FU means that it is already eluted by water itself, which in turn implies that extraction volumes have to be minimised, even though this may be to some degree compromised by sorbent mass. Fig. SM 2 (Supplementary material) shows how increasing sorbent mass improves the extraction efficiency of 5-FU and its internal standard 5-CU. Further, it is also evident that a more complex matrix such as wastewater has a notable negative impact on efficiency and repeatability of SPE. Based on these findings and considering the duration and costs of sample preparation we selected 500 mg/6 mL Isolute ENV+ cartridges to extract 5-FU from surface waters and 1 g/6 mL cartridges for wastewaters. As explained in Supplementary material 100 mg/3 mL Isolute ENV+ cartridges were selected for those biodegradation and photodegradation samples, which were performed at initial 5-FU concentrations of 1 ng/L and higher.

The optimised analytical method for determination of 5-FU uses 100 mL of wastewater or surface water at pH 6 and concentrates the analyte 667-fold, thus giving sufficient LOD concentrations of 0.48 ng/L for wastewater and 0.16 ng/L for surface water (Table 1). The LODs could be further decreased by switching from external to internal ionisation in the ion trap mass analyzer. Table 1 shows the results of the validation of analytical method for determination of 5-FU in wastewater and surface water samples.

According to the literature the quantification limits for 5-FU in wastewaters are 8.6 μg/L ([14,15]), 100 ng/L ([4,8]), and 50 ng/L when using negative chemical ionisation or 90 ng/L with EI ionisation [7]. The lowest reported LOQ was 5 ng/L obtained by Kovalova et al. [6]. Using our analytical method we were able to achieve sub ng/L (Table 1) LOQ. This is significant improvement to the state-of-the-art and may mean the difference between detecting or missing a target compound in a sample.

3.2. Determination of 5-FU in real water samples

Table 2 shows the results of 5-FU analyses in actual water samples taken during spring and autumn 2012. 5-FU was determined in
Table 1
Validation of analytical method for determination of 5-FU in wastewater and surface water samples.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Wastewater (matrix effect 99.6%)</th>
<th>Surface water</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPE efficiency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOD/LOQ (ng/L)</td>
<td>53 ± 28%</td>
<td>93 ± 2%</td>
</tr>
<tr>
<td>LOD: 0.48 ng/L; LOQ: 1.6 ng/L</td>
<td>0.966–0.993</td>
<td>0.947–0.997</td>
</tr>
<tr>
<td>Linearity (r²); conc. range 2–106 ng/L</td>
<td>14%</td>
<td></td>
</tr>
<tr>
<td>Repeatability of measurement</td>
<td>Conc. range 2–16 ng/L:</td>
<td></td>
</tr>
<tr>
<td>(3 injections)</td>
<td>Conc. range</td>
<td></td>
</tr>
<tr>
<td>Repeatability of analytical method (3 replicates)</td>
<td>5.20%</td>
<td></td>
</tr>
<tr>
<td>Accuracy error</td>
<td>10%</td>
<td>7.8%</td>
</tr>
</tbody>
</table>

4 out of 12 water samples. All positive samples were wastewaters. 5-FU was determined in concentrations up to 100 ng/L in wastewaters from oncological wards. Lower concentrations were found in the municipal wastewater treatment plant influents.

3.3. Photodegradation of 5-FU and CAP and identification of their phototransformation products

The UV degradation of 5-FU followed pseudo-first order kinetics with degradation rate constant of 0.045 min⁻¹ (r² = 0.98) and a half-life of 15 min. By adding a radical source such as H₂O₂ into the 5-FU solution in distilled water rapid removal was achieved, i.e. 99.6% of the parent compound was removed within 10 min of the UV/H₂O₂ treatment.

The chromatographic and mass spectrometric data on 5-FU are given in Table 3. For the MS/MS spectrum see the Supplementary material (Fig. SM 3). 5-FU with an elemental composition C₈H₇N₂O₂F shows a deprotonated molecule at [M–H]= 129.0. Its only ion fragment is found after cleavage of a CONH group at m/z 86.0, which corresponds to an elemental composition of C₈H₅N₂O₂F. During photodegradation few TPs form. Our attempts to identify these newly-formed compounds based on comparison of their mass spectra with those of the parent compound revealing the following TPs:

TP-147 elutes 0.2 min prior to 5-FU and shows a base fragment ion at m/z 104 and a deprotonated molecule at [M–H]= 147.0, which corresponds to the elemental composition of C₈H₅N₂O₂F (Table 3). This is, in comparison with the parent compound 5-FU, two protons and one oxygen atom higher. Analogous to 5-FU, the MS/MS of m/z 147 shows cleavage of CONH, which implies that this part of the molecule remained intact (Fig. 1) and that the additional oxygen is most likely attached to the unoccupied C-6 carbon, whereas the 5–6 double bond is saturated. Based on these data this TP is 5-fluoro-6-hydroxy-5,6-dihydroxycal (Fig. 1). This compound was first published back in 1964 by Lozzeron et al. [16], and was recognised as the main phototransformation product of 5-FU. In addition to TP-147, there is another photo-TP (ISO-TP-147, Table 3) that has approximately 22% abundance of TP-147 and shows identical MS/MS data. Miolo et al. [17] proposed that the addition of the hydroxyl group to 5-FU may occur on either side of the plane of the molecule, thus accounting for the formation of two isomers in which the fluorine and the oxygen are in cis or trans positions [17]. Accordingly, it is proposed that TP-147 and ISO-TP-147 are isomers formed following the photoaddition of water to the 5,6 double bond of 5-FU.

Furthermore, TP-127 shows a deprotonated molecule at [M–H]= 127.0, where its HR mass corresponds to C₄H₃N₂O₄. The cleavage of 43 Da [M–H–CONH]− at m/z 84 is also evident. This indicates that TP-127 was formed by defluorination and hydroxylation of 5-FU and the most likely structure of TP-127 is 5-hydroxyuracil. This phototransformation reaction was previously described for the fluorinated pharmaceutical citalopram [18]: insight into the reaction mechanism is given elsewhere [19].

TP-127-D elutes approximately 2 min after 5-FU and its TPs proposed above. Again, this compound has a base fragment ion at m/z 127 which corresponds to an elemental composition C₄H₃N₂O₄, and cleavage of 43 Da at m/z 84, which is common to all 5-FU analogues (Table 3). However, considering its chromatographic behaviour TP-127-D interacts more strongly with a C-18 stationary phase and it is therefore assumed that either the compound is less polar or has a higher molecular mass. Accordingly, careful inspection of its MS spectrum shows a very minor mass at m/z 253.0, presumably a deprotonated molecule of the 5-hydroxyuracil dimer. Its elemental composition was determined with rather high mass error of −6.9 mDa, which may be justified by its low abundance.

Analogous to TP-147 and ISO-TP-147 two more isomers were found (TP-143 and ISO-TP-143) with matching deprotonated molecules and MS/MS fragmentation. The [M–H]= 143.0 corresponds to C₄H₃N₂O₄, which is one oxygen extra to the elemental composition of TP-127 (Table 3), and it is therefore supposed that the hydroxyl group is attached to C-6 in cis and trans position. In this view, we assume that TP-143 and ISO-TP-143 are cis/trans 5,6-dihydroxycal isomers. The structures of the proposed 5-FU photo-TPs are incorporated into a breakdown scheme presented in Fig. 1.

CAP was found to be more resistant to UV degradation than 5-FU. The rate constant was 0.015 min⁻¹ (r² = 0.99) and a half-life 46 min. Fig. SM 5 (Supplementary material) shows how the

Table 2
Concentrations of 5-FU in Slovene surface and wastewater samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sampling approach</th>
<th>Sampling date</th>
<th>Origin</th>
<th>c (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospital A wastewater</td>
<td>Neutralisation</td>
<td>24.4.2012</td>
<td>Oncological ward (5-FU used in therapy)</td>
<td>92</td>
</tr>
<tr>
<td>Hospital A wastewater</td>
<td>Neutralisation</td>
<td>25.9.2012</td>
<td>Oncological ward (5-FU used in therapy)</td>
<td>35</td>
</tr>
<tr>
<td>Municipal wastewater A – WWTP influent</td>
<td>Composite: 24 h time proportional</td>
<td>16.3.2012</td>
<td>Municipal wastewater (360 000 PE) prior treatment</td>
<td>14</td>
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<td>Municipal wastewater A – WWTP effluent</td>
<td>Composite: 24 h time proportional</td>
<td>16.3.2012</td>
<td>Municipal wastewater (360 000 PE) post treatment</td>
<td>&lt;LOD</td>
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<td>Municipal wastewater A – WWTP effluent</td>
<td>Composite: 24 h time proportional</td>
<td>25.9.2012</td>
<td>Municipal wastewater (360 000 PE) post treatment</td>
<td>&lt;LOD</td>
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<tr>
<td>Municipal wastewater A – WWTP effluent</td>
<td>Composite: 24 h time proportional</td>
<td>16.3.2012</td>
<td>Municipal wastewater (360 000 PE) post treatment</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>Municipal wastewater A – WWTP effluent</td>
<td>Composite: 24 h time proportional</td>
<td>25.9.2012</td>
<td>Municipal wastewater (360 000 PE) post treatment</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>Municipal wastewater A – WWTP effluent</td>
<td>Composite: 24 h time proportional</td>
<td>16.3.2012</td>
<td>Municipal wastewater (360 000 PE) post treatment</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>River after WWTP A discharge</td>
<td>Grab</td>
<td>16.3.2012</td>
<td>Surface water: river</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>Hospital B wastewater – WWTP influent</td>
<td>Grab</td>
<td>26.9.2012</td>
<td>Oncological ward (5-FU NOT used in therapy)</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>Hospital B wastewater – WWTP effluent</td>
<td>Grab</td>
<td>26.9.2012</td>
<td>Oncological ward (5-FU NOT used in therapy)</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>Stream before hospital B discharge</td>
<td>Grab</td>
<td>26.9.2012</td>
<td>Surface water: stream</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>Stream after hospital B discharge</td>
<td>Grab</td>
<td>26.9.2012</td>
<td>Surface water: stream</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>Label</td>
<td>Retention time t&lt;sub&gt;R&lt;/sub&gt;</td>
<td>Elemental composition</td>
<td>MS/MS and HRMS based identification</td>
<td>MS or MS/MS fragment ions&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>-------</td>
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</tr>
<tr>
<td><strong>5-FU &amp; transformation products</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-FU</td>
<td>0.72 min</td>
<td>C₅H₁₀N₉O₇</td>
<td>[M − H]⁻ (C₅H₉N₉O₇F⁻/2.3 ppm) = 129.0097</td>
<td>m/z 86.0045 (C₅H₁₀NOF/3.5 ppm/ESI(−))</td>
</tr>
<tr>
<td>TP-147</td>
<td>0.48 min</td>
<td>C₅H₁₀N₉O₇</td>
<td>[M − H]⁻ (C₅H₉N₉O₇F⁻/0.0 ppm) = 147.0206</td>
<td>m/z 104.0150 (C₅H₉N₉O₇F⁻/1.9 ppm/ESI(−))</td>
</tr>
<tr>
<td>ISO-TP-147</td>
<td>0.66 min</td>
<td>C₅H₆N₉O₇</td>
<td>[M − H]⁻ (C₅H₅N₉O₇F⁻/−0.8 ppm) = 147.0189</td>
<td>m/z 104.0142 (C₅H₅N₉O₇F⁻/−5.8 ppm/ESI(−))</td>
</tr>
<tr>
<td>TP-127</td>
<td>0.55 min</td>
<td>C₅H₆N₉O₇</td>
<td>[M − H]⁻ (C₅H₅N₉O₇F⁻/−0.8 ppm) = 127.0143</td>
<td>m/z 84.0076 (C₅H₅NO₂⁻/−11.9 ppm/ESI(−))</td>
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<tr>
<td>TP-127-D</td>
<td>2.64 min</td>
<td>C₅H₆N₉O₇</td>
<td>[M − H]⁻ (C₅H₅N₉O₇F⁻/−27.3 ppm) = 253.0140</td>
<td>m/z 127.0146 (C₅H₅N₉O₇F⁻/1.6 ppm/ESI(−))</td>
</tr>
<tr>
<td>TP-143</td>
<td>0.42 min</td>
<td>C₅H₆N₉O₇</td>
<td>[M − H]⁻ (C₅H₅N₉O₇F⁻/−1.4 ppm) = 143.0091</td>
<td>m/z 84.0079 (C₅H₅NO₂⁻/−8.3 ppm/ESI(−))</td>
</tr>
<tr>
<td>ISO-TP-143</td>
<td>0.55 min</td>
<td>C₅H₆N₉O₇</td>
<td>[M − H]⁻ (C₅H₅N₉O₇F⁻/−1.4 ppm) = 143.0091</td>
<td>m/z 100.0032 (C₅H₅NO₂⁻/−3.0 ppm/ESI(−))</td>
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<tr>
<td><strong>CAP &amp; transformation products</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAP</td>
<td>4.16 min</td>
<td>C₆H₇N₉O₇</td>
<td>[M+H]+ (C₆H₇N₉O₇F⁺/0.3 ppm) = 360.1570</td>
<td>m/z 244.1094 (C₆H₇N₉O₇F⁺/−1.2 ppm)</td>
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<tr>
<td>ISO-CAP</td>
<td>4.30 min</td>
<td>C₆H₇N₉O₇</td>
<td>[M+H]+ (C₆H₇N₉O₇F⁺/0.5 ppm) = 382.1388</td>
<td>m/z 174.0325 (C₆H₇N₉O₇F⁺/5.7 ppm)</td>
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<tr>
<td>TPC-244</td>
<td>3.33 min</td>
<td>C₆H₁₄N₉O₇</td>
<td>[M+H]+ (C₆H₁₄N₉O₇F⁺/−2.9 ppm) = 244.1104</td>
<td>m/z 130.0424 (C₆H₁₄N₉O₇F⁺/5.4 ppm)</td>
</tr>
<tr>
<td>TPC-226</td>
<td>3.94 min</td>
<td>C₆H₁₄N₉O₇</td>
<td>[M+H]+ (C₆H₁₄N₉O₇F⁺/−3.5 ppm) = 266.0911</td>
<td>m/z 244.1094 (C₆H₁₄N₉O₇F⁺/−0.2 ppm)</td>
</tr>
<tr>
<td>TPC-242</td>
<td>3.09 min</td>
<td>C₆H₁₄N₉O₇</td>
<td>[M+H]+ (C₆H₁₄N₉O₇F⁺/−0.8 ppm) = 248.1009</td>
<td>m/z 156.0413 (C₆H₁₄N₉O₇F⁺/2.6 ppm)</td>
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<tr>
<td>TPC-358</td>
<td>3.89 min</td>
<td>C₆H₁₄N₉O₇</td>
<td>[M+H]+ (C₆H₁₄N₉O₇F⁺/−0.5 ppm) = 244.1104</td>
<td>m/z 112.0543 (C₆H₁₄N₉O₇F⁺/4.5 ppm)</td>
</tr>
<tr>
<td>TPC-378/L</td>
<td>3.75 min</td>
<td>C₆H₁₄N₉O₇</td>
<td>[M+H]+ (C₆H₁₄N₉O₇F⁺/−2.9 ppm) = 378.1666</td>
<td>m/z 172.0361 (C₆H₁₄N₉O₇F⁺/1.7 ppm)</td>
</tr>
<tr>
<td>TPC-378/M</td>
<td>3.95 min</td>
<td>C₆H₁₄N₉O₇</td>
<td>[M+H]+ (C₆H₁₄N₉O₇F⁺/−0.8 ppm) = 378.1674</td>
<td>m/z 262.1196 (C₆H₁₄N₉O₇F⁺/−2.7 ppm)</td>
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<td>TPC-378/R</td>
<td>4.57 min</td>
<td>C₆H₁₄N₉O₇</td>
<td>[M+H]+ (C₆H₁₄N₉O₇F⁺/−0.5 ppm) = 378.1675</td>
<td>m/z 192.0431 (C₆H₁₄N₉O₇F⁺/5.2 ppm)</td>
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<tr>
<td>TPC-378/A</td>
<td>4.84 min</td>
<td>C₆H₁₄N₉O₇</td>
<td>[M+H]+ (C₆H₁₄N₉O₇F⁺/0.5 ppm) = 378.1683</td>
<td>m/z 149.0359 (C₆H₁₄N₉O₇F⁺/−2.0 ppm)</td>
</tr>
<tr>
<td>TPC-260</td>
<td>2.81 min</td>
<td>C₆H₁₄N₉O₇</td>
<td>[M+H]+ (C₆H₁₄N₉O₇F⁺/−2.9 ppm) = 380.1435</td>
<td>m/z 134.0960 (C₆H₁₄N₉O₇F⁺/0.7 ppm)</td>
</tr>
<tr>
<td>5-FU</td>
<td>0.72 min</td>
<td>C₅H₁₀N₉O₇</td>
<td>[M − H]⁻ (C₅H₉N₉O₇F⁻/2.3 ppm) = 129.0103</td>
<td>m/z 217.0089 (C₅H₁₀N₉O₇F⁺/0.5 ppm/ESI(−))</td>
</tr>
</tbody>
</table>

<sup>a</sup> All ions are given in ESI(+) ionisation mode unless specified.
parent compound CAP is removed from the UV-irradiated solution. It remains present after 14 h of UV irradiation (data not shown); however, by adding H2O2 97% of CAP is removed within 10 min. It is further evident from Supplementary material (Fig. SM 5) that the decay of parent compound CAP is partially on account of a compound labelled as “ISO-CAP”, which rapidly emerges after 10 min of UV irradiation and was found to be the most abundant TP during first six hours of the UV treatment. Yet the total amount of CAP and ISO-CAP is nevertheless being decreased during the course of the UV irradiation experiment (see Fig. SM 5 in Supplementary material).

By extending the LC elution gradient CAP and at least 7 transformation products (TPs) appeared within 2.7–4.7 min (see Fig. 2). The MS and MS/MS spectra of the parent compound CAP are shown and described in detail in the Supplementary material (Fig. SM 6). Briefly, CAP elutes at 4.2 min and shows a protonated molecule at [M+H]+ = 360.2 (C15H23N3O8F), while its MS/MS fragments indicate cleavage of ribofuranose at m/z 244, further cleavage of penty1 chain at m/z 174, and finally dissociation of CO2 at m/z 130. ISO-CAP elutes 0.2 min after CAP (Fig. 2) and matches the parent compound in the MS and MS/MS fragmentation in ESI(+) ionisation mode (Table 3). One difference between the ISO-CAP and CAP mass spectra was found in the ESI(−) ionisation mode, where the ISO-CAP shows a minor ion fragment at m/z 270 (see Supplementary material Fig. SM 7), which corresponds to C10H9N3O5F and is formed after hydrolysis of the ester moiety with a cleavage of penty1 alcohol. This ion fragment is not observed for parent compound, which may be due to different spatial arrangement. Namely, CAP is a ribofuranose-based nucleoside, and has the stereochemical structure of a ribofuranose having a β-oriented 5-fluorocytosine moiety at the C1 position [20]. Considering the MS results it is proposed that ISO-CAP is a diastereomer of CAP, where most likely the β → α transformation occurs on the anomeric carbon.

One of most abundant TPs in Fig. 2 is TPC-244, which elutes at 3.3 min showing a protonated molecule at [M+H]+ = 244.1 and sodium adduct [M+Na]+ = 266.1. A dimer [2M+H]+ is also evident in its MS-TOF spectrum at 509.2 (Table 3). The elemental composition of the TPC-244 protonated molecule corresponds to the ion fragment of CAP m/z 244 (C10H16N3O4F) which is formed after the ribofuranose sugar is cleaved from the molecule. Furthermore, the fragmentation pattern of TPC-244 corresponds to that of the parent molecule CAP, which implies that TPC-244 is formed by photolytically-driven cleavage of the ribofuranose sugar. The chemical structure of TPC-244 is proposed in Fig. 3.

TPC-226 coelutes with another TP labelled TPC-378/M (Fig. 2), and was recognised based on its protonated molecule at [M+H]+ = 226.1 (C10H16N3O4), together with the sodium adduct at [M+Na]+ = 248.1. Comparison of the TPC-226 elemental composition to that of TPC-244 reveals that the compound lacks fluorine and holds additional hydrogen. Also the MS/MS fragmentation of TPC-226 is analogous to that of TPC-244, showing ion fragments at m/z 156 generated after a characteristic cleavage of a penty1 chain, and m/z 112 which forms by further dissociation of CO2. It is therefore assumed that TPC-226 is formed by defluorination of TPC-244 (see Fig. 3).

Another TP which seems to be strongly related to the previous two is TPC-242, that elutes at 3.1 min and shows a protonated molecule at [M+H]+ = 242.1 and a sodium adduct at [M+Na]+ = 264.1 (Table 3). The exact mass of the protonated molecule corresponds to C10H16N3O4, which in comparison to CAP elemental composition shows an absence of a fluorine atom and five carbons. The MS/MS spectrum of TPC-242 shows two major ion fragments, m/z 172 which stands for C7H6N3O5, and m/z 128 with an elemental composition of C6H3N3O5. Both ion fragments match the fragmentation pattern of the parent compound CAP showing the cleavage of the penty1 chain, which is followed by the cleavage of CO2. These data suggest that TPC-242 is altered in two ways: first, the compound lacks the ribofuranose segment and second, the fluorine atom is replaced by a hydroxyl group. The cleavage of fluorine and hydroxylation is a two-step UV transformation reaction which is also typical for 5-FU (Fig. 1). The chemical structure of TPC-242 is proposed in Fig. 3. The breakdown pathway of TPC-244, TPC-242 and TPC-226 is revealed based on their degradation curves (Supplementary material, Fig. SM 8). First the ribofuranose component is removed, which makes TPC-244 a primary photo-transformation product. Since TPC-226 reaches its maximum at

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**Fig. 1.** UV breakdown scheme of 5-FU.
Fig. 2. LC separation of parent compound CAP and its phototransformation products.

Fig. 3. UV breakdown scheme of CAP.
approximately 6 h of UV irradiation, while the concentration of TP-242 is still increasing; we suppose that TP-244 is first defluorinated to form TPC-226, which is in the next step hydroxylated into TPC-242.

TPC-358 is a minor TP coeluting with TPC-226 and TPC-378/M (Fig. 2). Its protonated molecule at [M+H]* = 358.2 (C13H23N2O7) along with the sodium adduct [M+Na]* = 380.1 implies that the fluorine atom is replaced by an oxygen, so that the defluorination and hydroxylation occurs as depicted for the transformation reactions 5-FU → TP-127 and TPC-244 → TPC-226 → TPC-242. This was confirmed by the analogous fragmentation pattern of the TPC-358 and its desugared analogue TPC-242 (Table 3). Considering the transformation TPC-244 → TPC-226 → TPC-242 one would expect to find also a defluorinated TPC-226 analogue at [M+H]* = 342.2 with proposed elemental composition C13H22N2O6. Despite targeted screening for such TP we failed to detect it, which might be reasoned by its amount being insufficient for detection. This is further supported by the fact that neither TPC-358, nor TPC-226 was abundant.

The chromatogram of UV-irradiated CAP (Fig. 2) reveals at least three TPs with a protonated molecule at [M+H]* = 378.2 with elemental composition of C13H23N2O7. Further, sodium adduct at [M+Na]* = 402.0 is evident for each of these TPs. The compounds are labelled as TPC-378/L (left), TPC-378/M (middle), TPC-378/R (right) according to their retention times. In comparison to the elemental composition of the parent compound this transformation indicates the addition of oxygen and two protons. The MS/MS spectrum of TPC-378/L shows a rich fragmentation pattern (Fig. SM 9, Supplementary material). Analogous to CAP first the 116 Da ribofuranose (C4H6O3) is cleaved forming m/z 262, and is followed by dissociation of pentyl chain at m/z 192. In contrast with CAP cleavage of CONH (43 Da) is found, which is a cleavage characteristic for 5-FU (Table 3) and forms an abundant ion at m/z 219 (C9H16N2O2F) in ESI(+) or m/z 217 (C9H16N2O2F) in ESI(–). Another crucial ion that facilitated the identification of TPC-378/L is m/z 129 found in its ESI(–) MS/MS spectrum, which has the identical elemental composition as the 5-FU deprotonated molecule (Fig. SM 11, Supplementary material). Based on the MS and MS/MS data we suppose that CAP was the subject of the same phototransformation reaction as 5-FU, i.e. the photoaddition of water to 5-fluoropyrimidin-2-(1H)-one segment in CAP molecule. The deolocalisation of the double bond is possible as evident in Supplementary material (Fig. SM 12, Supplementary material). The chemical structure of TPC-378/L is proposed in Fig. 3. TPC-378/R and TPC-378/M show the identical fragmentation pattern as TPC-378/L and are therefore supposed to be the diastereomers as already described for CAP and ISO-CAP as well as for two 5-FU isomers formed by photoaddition of water (TP-147 and ISO-TP-147).

As evident from Table 3 and Fig. 2 TPC-260 emerges at 2.81 min as an abundant sodium adduct peak at [M+Na]* = 260.1, which corresponds to the elemental composition of C9H16N2F2Na, whereas TPC-260 protonated molecule at [M+H]* = 238.1 is barely seen. Further, a [2M+Na]* adduct is evident in its mass spectrum at 497.2. The mass spectrum of TPC-260 does not contain ion fragments common to the parent compound CAP, which suggests that several structural alterations occurred on this molecule. Taking into account possible alterations on chemical structure of CAP (Fig. 3) a segment of the molecule containing 9 carbon atoms can be preserved after detachment of ribofuranose moiety and cleavage of urea from the pyrimidine ring and concurrent oxidation of the former two pyrimidine C=C bond. TPC-260 molecule was found very resistant to MS/MS fragmentation resulting in few minor ion fragments including m/z 220 formed after initial detachment of water molecule, m/z 150 (C4H8NO3F) that stands for characteristic cleavage of 70 Da pentyl chain, m/z 106 (C9H16N2O2F) formed after further cleavage of CO2, and finally by loss of a second water molecule m/z 88 (C13H2NOF) is formed. The chemical structure of TPC-260 is proposed in Fig. 3, however, the C=O double bond may as well be de-localised and the position of one hydroxyl group is not certain, even though it is mostly located on the first carbon due to lowest steric hindrances.

By targeted screening in ESI(–) mode we also determined 5-FU as a phototransformation product of CAP. The compound was confirmed by matching its retention time and MS data with the authentic standard (Table 3).

3.4. Biodegradation of 5-FU and CAP

Batch biodegradation experiments at the Erlenmeyer scale were carried out aiming to reveal the biodegradability of 5-FU and CAP and to characterise the biodegradation products formed. At an AS concentration of 0.67 g/L 99.99% of 5-FU was eliminated from the test mixture within 40 h with estimated half-life biodegradation time of 8 h (see Table-SM 7 in Supplementary material). By decreasing the AS concentration by 5 and 50-fold the biodegradation of 5-FU slowed down reaching 7.1 and 16 h for 0.14 and 0.014 g/L AS, respectively. A graph revealing the removal of 5-FU in respect to the AS concentration is shown in Fig. 4.

A limited number of studies are available on the biodegradation of 5-FU and reveal contradictory results regarding the potential for biodegradation of 5-FU [2,4]. In agreement with our results Kiffmeyer et al. [21] and Mahnkopf et al. [15] suggest rapid biodegradation of 5-FU. In contrast Kummerer and Al-Ahmad [22] and Yu et al. [8] showed that 5-FU was persistent to biodegradation. Straub [4] critically evaluated available data regarding 5-FU biodegradability tests and suggested that biodegradability of 5-FU
was concentration-dependent, which is based on the assumption that high concentrations may be having cytotoxic effects on the degrading microorganisms potentially leading to false negative results [23]. 3rd biodegradation experiment results show that by increasing the concentration of 5-FU to 10 mg/L and 20 mg/L there is a lag phase of approximately 12 h, but 5-FU eventually degrades.

Conversely, this is not the case at 100 mg/L of 5-FU, where no biodegradation is observed within 43 h. The finding that 5-FU in higher concentrations is persistent to biodegradation may justify the contradictory results on biodegradability of 5-FU in the literature, where most of the experiments were conducted in the concentration range 9–854 mg/L [4].

Straub [4] further reviewed the biodegradability of CAP showing that the compound was slowly degraded. Since biodegradability of 5-FU and CAP was conducted by different tests in different laboratories it is difficult to compare biodegradation rates. In the present study we performed a biodegradability test for CAP under identical conditions as for 5-FU and found that CAP was far more persistent in comparison to the active drug. In this sense, 1/2 of 77 h was determined for CAP and >99% removal was achieved in more than 11 days, whereas 5-FU was under the same conditions >99% degraded within 40 h. By decreasing the AS concentration by 5 and 50-fold we were not able to achieve >99% removal of CAP during the course of the biodegradation experiment. The results of 5-FU and CAP biodegradation under different conditions are comprised in Supplementary material (Table-SM 7).

By untargeted screening we did not detect any bio-TPs in either 5-FU or the CAP samples. However, by targeted screening trace amounts of 5-FU were detected in the CAP biodegradation samples. The failure to detect other bio-TPs may be on account of their low concentrations. Another possible reason is that bio-TPs are more biodegradable than the parent compound (like 5-FU is more biodegradable than CAP), which makes the parent compound a bottleneck in biodegradation, whereas bio-TPs, when formed, rapidly disappear from solution.

4. Conclusions

This study highlights the analysis, environmental occurrence, fate and transformation of an anticancer drug 5-FU. 5-FU is a polar low molecular mass compound and is thus demanding to extract and analyse in aqueous matrices, particularly when it occurs in trace levels. While ion pair and anion exchange retention mechanisms are promising alternatives for simple matrices, reversed phase SPE using a very hydrophilic hyper-cross-linked hydroxylated polystyrene divinylbenzene copolymer sorbent was found to be the only viable option for preparation of wastewater samples. The presented analytical method for determination of 5-FU in natural and wastewater samples reports highest sensitivity published so far. Besides, this is the first study to determine 5-FU in municipal wastewaters in concentrations as low as 4.7 and 14 ng/L, while in hospital wastewaters the levels of this analyte were higher (35 and 92 ng/L).

Further, for the first time biodegradability and photodegradability of 5-FU and its prodrug CAP are addressed. In general, CAP was found to be more resistant to both degradation processes, and it is therefore assumed that this compound will be more persistent in the environment. Yet, degradation processes often do not lead to the complete mineralisation; instead breakdown products emerge, which as well as the parent compounds represent environmentally relevant xenobiotics. As a result of simulated photodegradation experiments we found a number of transformation products. Among them we propose chemical structures for six 5-FU and ten CAP transformation products. There were also several TPs that could not be identified, which was either due to their insufficient abundance, chromatographic coelution or instrumental limitations. These compounds should be investigated further, possibly by using alternative separation and identification techniques. Most common phototransformation reactions were photoaddition of water, defluorination and hydroxylation; commonly diastereomeric photoproducts were formed. As virtually no information on the transformation of anticancer drugs is currently available, the proposed TPs bring an important contribution to recognising the environmental fate of 5-FU and CAP. In addition, these compounds might have clinical significance, since the improvement of efficacy of 5-FU in the treatment of certain solid tumours and advanced breast cancers [17] may be attributed to photo-TPs. Finally, because of the increasing attention given to the qualitative determination of pharmaceutical degradation products in the literature and the development of identification tools in general, knowledge about the fate of this particular group of pharmaceuticals in the environment will become more comprehensive.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.03.046.

References
