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Environmental Toxicology and Chemistry, Vol. 23, No. 6, pp. 1431–1440, 2004

Printed in the USA
0730-7268/04 $12.00 + .00

AQUATIC PERSISTENCE OF EIGHT PHARMACEUTICALS IN A MICROCOSM STUDY

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(*Received* 23 *July* 2003; *Accepted* 21 *November* 2003)

Abstract—The persistence of eight pharmaceuticals from multiple classes was studied in aquatic outdoor field microcosms. A method was developed for the determination of a mixture of acetaminophen, atorvastatin, caffeine, carbamazepine, levofloxacin, sertraline, sulfamethoxazole, and trimethoprim at μg/L levels from surface water of the microcosms using solid phase extraction and high-performance liquid chromatography-ultraviolet (HPLC-UV) and liquid chromatography tandem mass spectrometry (LC-MS-MS). Half-lives in the field ranged from 1.5 to 82 d. Laboratory persistence tests were performed to determine the relative importance of possible loss processes in the microcosms over the course of the study. Results from dark control experiments suggest hydrolysis was not important in the loss of the compounds. No significant differences were observed between measured half-lives of the pharmaceuticals in sunlight-exposed pond water and autoclaved pond water, which suggests photodegradation was important in limiting their persistence, and biodegradation was not an important loss process in surface water over the duration of the study. Observed photoproducts of several of the pharmaceuticals remained photoreactive, which led to further degradation in irradiated surface waters.

Keywords—Pharmaceuticals Persistence Liquid chromatography tandem mass spectrometry Photodegradation High-performance liquid chromatography-ultraviolet

INTRODUCTION

The occurrence and fate of pharmaceuticals for human and veterinary use in the aquatic environment has become a subject of substantial scientific and public concern [1–3]. Several physiologically active compounds, such as caffeine, nicotine, and acetylsalicylic acid [4], have been known for two decades to enter the environment by a variety of routes, especially in more populated areas. Only recently with the development of more sensitive analytical techniques did it become evident that many drugs and personal care products, covering a wide spectrum of therapeutic use for humans and animals, could be inadvertently released into the environment. Entry into surface water can occur by a variety of routes, but primarily via treated and untreated sewage effluent and terrestrial run-off from agricultural communities.

More than 40 pharmaceuticals have been identified in sewage effluents and surface waters ranging from pg/L to μg/L level [1–3,5]. Recent U.S. Geological Survey (U.S. GS) data found a median of 7 and as many as 38 detectable organic wastewater contaminants present at μg/L concentrations in water samples taken from 139 streams across 30 states [6]. However, the effects and persistence of many of these pharmaceutical substances in the aquatic environment remains largely unknown. Pharmaceuticals have the potential to be more persistent than other organic pollutants, such as pesticides, because their source continually replenishes any removal by environmental processes such as microbial degradation, photolysis or particulate sorption. Consequently, pharmaceuticals that may degrade quickly would effectively behave as persistent compounds due to their constant release into the aquatic environment.

The guidelines given by the U.S. Food and Drug Administration Center for Drug Evaluation and Research to industry in stress testing of new drugs include determining the stability of drugs to light, temperature, and pH changes before their manufacture and prescription to humans or animals is permitted [7]. Although the photosensitivity of several fluoroquinolones [8,9], sulfamethoxazole [10,11], and trimethoprim [12] previously have been studied, there are a limited number of studies investigating the effects of pharmaceutical mixtures to nontarget organism and their fate in aquatic environments into which these organic wastewater contaminants are discharged [13–15].

The ecotoxicological effects and environmental persistence of the following eight pharmaceuticals were investigated in a microcosm study: Acetaminophen (ACT), caffeine (CAF), sulfamethoxazole (SMX), trimethoprim (TRPRM), levofloxacin (LEVO), sertraline (SRT), atorvastatin (ATR), and carbamazepine (CAR) (see Fig. 1 for structures). Acetaminophen, CAF, SMX, and TRPRM were among the list of detected organic wastewater contaminants in the U.S. GS survey, and LEVO and SRT belong to the same family as two of the detected drugs (e.g., fluoroquinolone and selective serotonin reuptake inhibitors). Carbamazepine is an antiepileptic that has been detected in European surface waters [16,17], and atorvastatin is one of the most prescribed drugs in the United States today (http://www.rxlist.com); both have the potential to be organic wastewater contaminants.

In order to determine effects of chronic exposure of the eight-pharmaceutical mixture to various aquatic organisms used in the microcosm study, the drugs needed to be maintained at a constant concentration. Adjustments to the phar-

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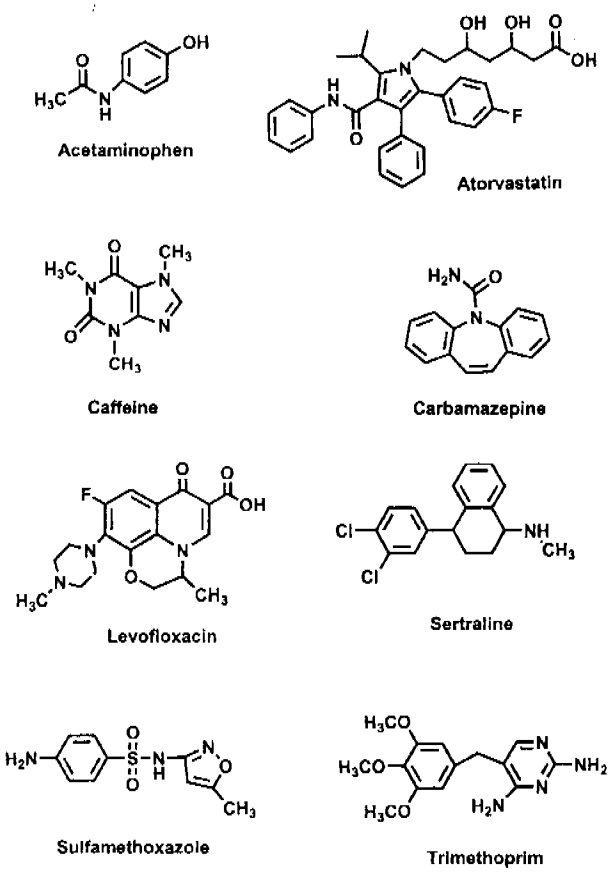

Fig. 1. Structures of pharmaceuticals used in study.

maceutical concentrations were based on laboratory quantification. Thus, water samples that were taken routinely required rapid and accurate analysis so that drug concentrations in the microcosms could be renewed appropriately to adjust for their possible losses. Because most pharmaceuticals have been observed to occur at μg/L levels in sewage treatment plant effluents, the microcosms were treated with environmentally realistic concentrations as well as greater concentrations to elicit quantifiable biological effects of the selected drugs. Treatment concentrations were chosen based on distributional analyses of upper exposure centiles (e.g., 95th, 99th, 99.5th, and 99.9th) estimated from actual surface water concentrations when available; otherwise exposure concentrations were set at 1, 10, 100, and 300 μg/L [15].

The objectives of this study were to develop a rapid, simple, reliable, and robust analytical method involving solid phase extraction (SPE) and high-performance liquid chromatography-ultraviolet (HPLC-UV) and liquid chromatography tandem mass spectrometry (LC-MS-MS) using positive electrospray (ES) for the analysis of a mixture of pharmaceuticals present at μg/L levels and to investigate the persistence of these compounds in an aquatic environmental setting.

MATERIALS AND METHODS

Chemicals

All chemicals were analytical reagent grade and were used as received. Acetaminophen (99%), caffeine (98.9%), carbamazepine (99.5%), and sulfamethoxazole (100%) and ammonium formate were obtained from Sigma (Oakville, ON, Canada). Atorvastatin (99%) was obtained from Rugao (Shanghai, China). Levofloxacin (90.8%) was obtained from Zhejiang Wonderful Pharma & Chemical (Zhejiang, China). Sertraline (98%) was obtained from Ranbaxy Laboratories (New Delhi, India). Trimethoprim (98.5%) was obtained from Fluka (Oakville, ON, Canada). Potassium monophosphate was purchased from ACP (Montreal, PQ, Canada). The *o*-phosphoric acid and formic acid were obtained from BDH (Toronto, ON, Canada). Optima grade acetonitrile purchased from Fisher (Nepean, ON, Canada) was used for LC-MS-MS analyses; HPLC–grade acetonitrile (>99%) from Caledon (Georgetown, ON, Canada) was used for HPLC-UV analyses; and Barnstead E-pure water with a resistivity of 18 MΩcm^{-1} was used for buffer preparation. Analytical stock solutions of the pharmaceuticals were made up in an appropriate solvent and were stored in the dark at 3°C.

Table 1. Treatment concentrations used in low, medium, high, and ultra high treatment level microcosms

Compound[a]	LT[b] (μg/L)	MT[c] (μg/L)	HT[d] (μg/L)	UHT[e] (μg/L)
ACT	0.83	33.98	132.00	2190
ATR	1.00	10.00	100.00	300.00
CAF	2.39	20.93	46.00	238.57
CAR	1.00	10.00	100.00	300.00
LEVO	1.00	10.00	100.00	300.00
SRT	1.00	10.00	100.00	300.00
SMX	0.31	6.31	19.00	182.78
TRPRM	0.20	0.81	1.36	3.97

[a] ACT = acetaminophen; ATR = atorvastatin; CAF = caffeine; CAR = carbamazepine; LEVO = levofloxacin; SRT = sertraline; SMX = sulfamethoxazole; TRPRM = trimethoprim.
[b] LT = low treatment.
[c] MT = medium treatment.
[d] HT = high treatment.
[e] UHT = ultra high treatment.

Solid phase extraction

Microcosm pond water samples containing pharmaceuticals at the concentrations shown in Table 1 were extracted using Oasis Hydrophilic-Lipophilic Balance C18 SPE cartridges (3 cc, 60 mg) (Waters, Milford, MA, USA) on a vacuum extraction manifold. The volume taken for extraction ranged from 20 to 50 ml, depending on the treatment level of the microcosm. Each SPE cartridge was conditioned with three column volumes each of methanol and deionized water. Samples were then passed through at a flow rate of 1 ml/min. The cartridges next were washed with 1 column volume of 5% methanol and dried for 10 min before the adsorbed compounds were eluted with 500 μl methanol followed by 500 μl methanol acidified with acetic acid. The low, medium, and high treatment (LT, MT, and HT) level ponds required 50:1, 20:1, and 20:1 concentration, respectively. Ultra high treatment (UHT) level samples were analyzed directly.

Analytical methods

Ultraviolet-vis absorption spectra of the pharmaceuticals were recorded using a Hewlett-Packard 8453A diode array spectrophotometer (Avondale, PA, USA). The HPLC analyses of samples taken from HT and UHT microcosms were performed at room temperature using a Waters 600S pump fitted with a reverse phase column (Alltima C-18 5 μm, 250 × 4.6 mm). Duplicate 100-μL injections of each sample were made using a Waters 717 autosampler. Target compounds were de-

tected and quantified using a Waters 996 photodiode array detector at the following wavelengths: 214 nm (SRT), 240 nm (ATR), 260 nm (ACT), 274 nm (CAF, SMX, TRPRM), and 290 nm (CAR, LEVO). A flow rate of 1.2 ml/min was used for all analyses. Separation of all drugs was achieved using a gradient program with a mobile phase consisting of acetonitrile and a potassium monophosphate buffer (40 mM adjusted to pH 3 using *o*-phosphoric acid). A starting composition of 15% acetonitrile:85% buffer was held for 6 min, then the acetonitrile composition was increased to 40% over 2 min and held for 10 min. Acetonitrile composition subsequently was increased from 40% to 75% over 1 min and held for an additional 5 min. Amounts were quantified by external calibration at the appropriate wavelength.

Because pharmaceutical concentrations in the LT and MT microcosms were below the limit of detection of the HPLC-UV system, these samples were analyzed by LC-MS-MS after sample concentration using the extraction procedure described above. The LC-MS-MS system consisted of a Waters 616 pump, 600 controller (Waters), and a Thermasphere TS-430 HPLC column heater/chiller (Phenomenex, Torrance, CA, USA) connected to a Micromass quattro micro LC tandem mass spectrometer with Z Spray® ES source operating in positive MS-MS mode (Micromass UK, Cheshire, UK). Chromatographic separations were performed at 30°C using an Allure C18 column (50 mm × 2.1 mm, 5 μm) (Chromatographic Specialties, Brockville, ON, Canada) preceded by a C18 guard cartridge (Phenomenex). The mobile phase eluents were acetonitrile and an aqueous ammonium formate buffer (10 mM adjusted to pH 3 with formic acid). The mobile phase was delivered to the HPLC column at a flow rate of 200 μl/min. A gradient elution was performed to separate and elute the analytes of interest (0–5 min, 15% acetonitrile; 8 min, 50% acetonitrile; 9–17 min, 90% acetonitrile). Sample injections were made using a Waters 717 autosampler. The Quattro Micro instrument was calibrated with a solution of sodium iodide according to the manufacturers' instructions. Tuning was performed for each analyte of interest by direct infusion of a 1-ng/μl solution at a flow rate of 20 μl/min using a Hamilton syringe (500 μl, Reno, NV, USA). The MS-MS parameters used for the analysis of all compounds is as follows: Capillary voltage, 3.00 kV; source temperature, 110°C; desolvation temperature, 225°C; gas flow, 250 L/h; resolution (LM1, HM1, LM2, HM2) 13.5; ion energy 1 and 2, 0.6; entrance 2,=; exit, 3; multiplier, 650 V; dwell time, 0.5 s. The optimal cone voltage and collision energy, corresponding to a (nearly) 100% fragmentation of the molecular ions, were different for each analyte and are summarized in Table 2. For quantification, the instrument was operated in multiple reaction monitoring mode, using for each analyte one precursor ion > product ion transition as shown in Table 2. Quantification was based on external calibration and was done with the MassLynx software (Micromass UK) using the above-mentioned multiple reaction monitoring mode transitions mentioned in Table 2.

Method validation

The method limit of quantification (LOQ) of each compound for both methods was established by extracting and analyzing at least five spiked blank samples at the LT and HT levels with the lowest concentration of each pharmaceutical used to construct calibration graphs. The method limit of detection (LOD) was defined as the lowest concentration of each pharmaceutical that could be recognized by the detector with a signal-to-noise ratio of ≥3. Spike and recovery experiments were conducted to evaluate the efficiency of the extraction procedure. To further evaluate the methods, five standard additions were done to water samples containing MT and HT concentrations, which subsequently were extracted and analyzed.

Table 2. Optimum cone voltage, collision energy, and parent and daughter ions monitored for the tandem mass spectrometry determination of pharmaceuticals

Compound[a]	Cone voltage (V)	Collision energy (eV)	Parent ion (*m/z*)	Daughter ion (*m/z*)
ACT	33	25	152	110
ATR	32	20	559	440
CAF	34	25	195	138
CAR	32	20	237	194
LEVO	34	20	362	318
SRT	15	10	308	277
SMX	29	15	254	156
TRPRM	40	25	291	123

[a] ACT = acetaminophen; ATR = atorvastatin; CAF = caffeine; CAR = carbamazepine; LEVO = levofloxacin; SRT = sertraline; SMX = sulfamethoxazole; TRPRM = trimethoprim.

Ambient photoperiod persistence study

Approximately 4 L of water from the control microcosm pond was obtained at the start of the microcosm study. The Pyrex® (Corning, NY, USA) bottles and lids that were used in the persistence study and about half of the control pond water were autoclaved for 90 min at 120°C. The same eight pharmaceuticals used in the microcosm study were spiked at μM levels into the Pyrex bottles that held either control microcosm pond water or autoclaved control microcosm water. Solutions were exposed to sunlight on the roof of the Lash Miller building at the University of Toronto (Toronto, ON, Canada) for 30 d in August. Dark control experiments also were conducted at this time. All experiments were done in duplicate. Sample aliquots from each Pyrex bottle were taken periodically over the course of the study and immediately analyzed quantitatively by the HPLC-UV method described above (see *Analytical methods*, this section) to determine the amount of the compound of interest remaining after solar irradiation based on external calibration. Pseudo-first order rate constants, k_{obs}, for the model compounds were obtained by linear regression of logarithmic concentration values determined as a function of time.

Microcosm and persistence study

Fifteen 12,000 L microcosms containing aquatic communities at the Centre for Toxicology Microcosm Research Facility located at the University of Guelph (Guelph, ON, Canada) were used for this study [18]. The microcosms contained communities of fish, aquatic plants, zooplankton, phytoplankton, macrophytes, and bacteria. Four mixtures of eight pharmaceuticals of varying concentrations ranging from low, medium, high, and ultra high treatment levels (see Table 1) were added into the appropriate ponds in triplicate, with three ponds designated as controls. Ponds were sampled and retreated based on laboratory quantification every three to four d in order to mimic constant infusion of the drugs and to keep levels steady for toxicological studies. The microcosm samples were extracted and analyzed for the amount of parent com-

Table 3. Instrument linearity range, method limit of quantification, method limit of detection, and extraction efficiency for the pharmaceuticals by high-performance liquid chromatography ultra violet (UV) and liquid chromatography tandem mass spectrometry

Compound[a]	Instrument range of linearity (μg/L)		Method LOQ[b] (μg/L)		Method LOD[c] (μg/L)		Average extraction efficiency (%) $n = 10$
	HPLC-UV[d]	LC-MS-MS[e]	HPLC-UV[d]	LC-MS-MS[e]	HPLC-UV[d]	LC-MS-MS[e]	
ACT	100–3,960	2–1,000	5.0	0.04	1.0	0.02	97.9 ± 0.6
ATR	150–4,000	0.5–300	7.5	0.01	0.9	0.004	76.9 ± 1.1
CAF	60–1,840	3–1,000	3.0	0.06	0.9	0.04	97.0 ± 0.4
CAR	75–4,000	0.06–1,000	3.8	0.001	0.5	0.001	95.9 ± 0.1
LEVO	150–4,000	1–300	7.5	0.02	1.9	0.01	95.9 ± 0.2
SRT	150–4,000	0.5–300	7.5	0.01	1.9	0.004	98.2 ± 3.0
SMX	91–3,120	1–300	4.6	0.02	0.3	0.01	89.0 ± 1.3
TRPRM	NA[f]	0.5–150	NA	0.01	NA	0.006	93.6 ± 1.8

[a] ACT = acetaminophen; ATR = atorvastatin; CAF = caffeine; CAR = carbamazepine; LEVO = levofloxacin; SRT = sertraline; SMX = sulfamethoxazole; TRPRM = trimethoprim.
[b] LOQ = limit of quantification.
[c] LOD = limit of detection.
[d] HPLC = high-performance liquid chromatography.
[e] LC-MS-MS = liquid chromatography tandem mass spectrometry.
[f] NA = Not applicable.

pound remaining by the methods described above. At the end of the microcosm study, the ponds were treated with the appropriate concentrations one last time and samples were taken at predetermined time points to determine field persistence. Pseudo-first order rate constants, k_{obs}, for the pharmaceuticals were determined from linear regression of logarithmic concentration values determined as a function of time.

Photodegradation study

For the photolysis experiment, 1-μM solutions of atorvastatin, carbamazepine, levofloxacin, and sulfamethoxazole were prepared separately in pure water. Fifty ml of each solution was placed in a quartz photochemical tube and irradiated in a sunlight simulator (Atlas CPS Sunlight Photosimulator, Chicago, IL, USA). Sample aliquots were taken at regular time intervals and analyzed by HPLC-UV for the amount of parent compound remaining. After the passage of at least two half-lives of the pharmaceuticals, the remaining solution was concentrated by SPE and the eluate was infused to the mass spectrometer to identify photoproducts. Daughter ion scans were performed for degradates observed and the appropriate transitions were monitored in the sample aliquots taken during an ensuing photolysis experiment.

RESULTS AND DISCUSSION

Method validation

Calibration curves obtained from the analysis of standards for all eight pharmaceuticals using HPLC-UV and LC-MS-MS were linear over the ranges tested (see Table 3). Each calibration curve gave r^2 values ≥ 0.99. The regression slopes obtained using LC-MS-MS, however, showed between-day variation in the sensitivity of the instrument. The response to the same standard could vary as much as 30%. Hence, it was necessary to construct a new calibration curve daily before and after sample analysis with freshly prepared standards. The methods also were shown to be extremely precise, as the majority of relative standard deviations for duplicate injections were <3%. The method LOQ and LOD of the pharmaceuticals using the SPE, HPLC-UV, and LC-MS-MS methods described above (see *Materials and Methods* section) are listed in Table 3. The method LOQ and LOD concentrations are based on typical procedures that used a 20-ml (MT and HT) or 50-ml (LT) water sample and a final concentration of analyte in 1 ml with an injection volume of 20 μl and 100 μl for LC-MS-MS and HPLC-UV analyses, respectively. An instrument linearity range, method LOQ or LOD were not determined for trimethoprim using the HPLC-UV method because the microcosm treatment concentrations in the UHT and HT ponds were lower than the instrument detection limit for this pharmaceutical.

Spike and recovery experiments were performed by spiking known concentrations of the pharmaceuticals into control microcosm pond water and extracting and analyzing for the target compounds using the HPLC-UV and LC-MS-MS conditions described above to evaluate the efficiency of the extraction procedure. The concentrations used in these experiments were the same as the treatment concentrations used in the microcosm study. Ten sample replicates for the recovery experiments at each treatment level were performed. Average recoveries typically were greater than 75%, with the majority being greater than 95% (see Table 3). Little variation was observed between replicate extractions.

Five standard additions of each pharmaceutical were performed into prepared water samples containing MT and HT concentrations. These samples were then extracted and analyzed using the procedures described above (see *Materials and Methods* section). The constructed standard addition curves for the pharmaceuticals typically had $r^2 \geq 0.99$, but the experimentally obtained concentrations of the analytes typically were lower than their true values, which is likely the result of signal suppression from matrix effects. Further evidence of matrix effects come from the lower extraction efficiencies for compounds in field water compared to pure water as observed from spike and recovery experiments.

Chromatography

Representative chromatograms obtained using HPLC-UV and LC-MS-MS are shown in Figure 2a,b. Acetaminophen, caffeine, trimethoprim, and levofloxacin eluted quickly while sulfamethoxazole, carbamazepine, sertraline, and atorvastatin were retained longer. The use of a step function rather than a smooth gradient reduced the retention times of the more strongly retained compounds so that all analytes were eluted in less than 26 min by HPLC-UV and 17 min by LC-MS-MS.

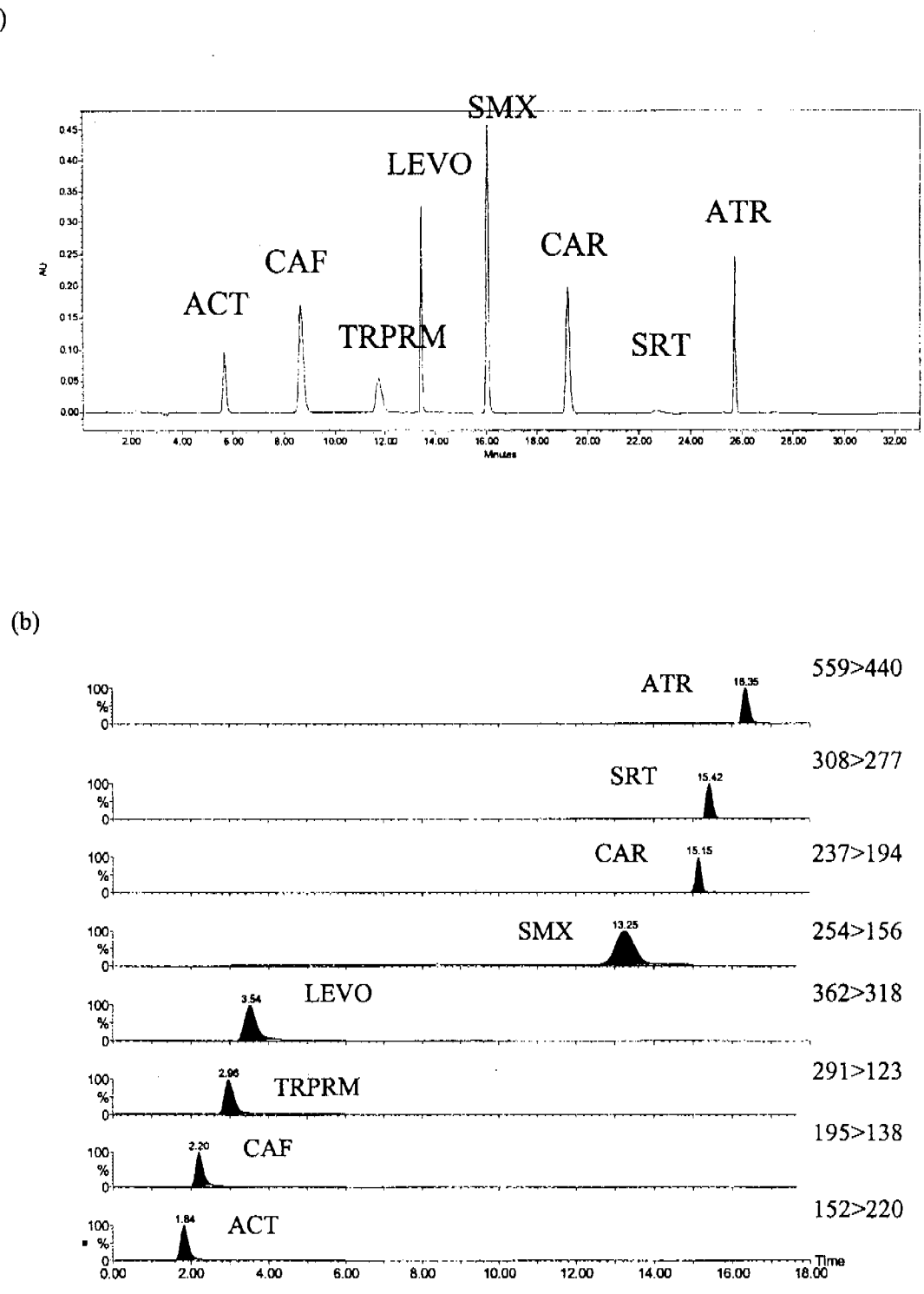

Fig. 2. Chromatogram of pharmaceutical mixture obtained by (a) high-performance liquid chromatography-ultraviolet at 274 nm and (b) liquid chromatography tandem mass spectrometry (ACT = acetaminophen; ATR = atorvastatain; CAF = caffeine; CAR = carbamazepine; LEVO = levofloxacin; SRT = sertraline; SMX = sulfamethoxazole; TRPRM = trimethoprim).

Good resolution of the compounds of interest was achieved using gradient elution in HPLC-UV analysis. The use of a diode array detector allowed for the simultaneous detection and quantification of compounds at different wavelengths. A formate-based buffer was used for LC-MS-MS gradient elutions because the phosphate buffer used for HPLC-UV analyses was unsuitable for use with electrospray mass spectrometry (MS) due to its lower volatility. The selectivity of the MS-MS detector allowed coeluting compounds to be detected separately by monitoring different ions simultaneously, and complete peak resolution was unnecessary. Because the less retained pharmaceuticals eluted in less than 4 min, their re-

spective parent–daughter transitions were only scanned for in the first 5 min of the analysis, and the transitions for the remaining four compounds were monitored for in the window in which they elute (12–17 min). Reducing the number of transitions being scanned simultaneously increased the sensitivity of the spectrometer toward each transition at low concentrations.

Mass spectrometry

The positive ion mode was selected on the basis of the presence of a basic functionality in the chemical structure of all the pharmaceuticals.

With the MS-MS parameters used, the parent–daughter transition of 152 > 110 for acetaminophen corresponded to its molecular ion and the fragment ion $^{+}NH_3$-C_6H_4-OH obtained after an $OC{=}CH_2$ neutral loss. The cleavage of C_6H_5-NCO from the molecular ion of atorvastatin with a *m/z* of 559 resulted in a 559 > 440 transition for this compound. The monitored precursor and fragment ions for caffeine were 195 and 138, respectively, where the fragment ion was obtained following the loss of H_3C-$N^{\cdot}$-$C^{\cdot}$=O. For carbamazepine the *m/z* value of 237 corresponded to its molecular ion, and the neutral loss of NH=C=O resulted in a fragment ion with a *m/z* = 194. The loss of CO_2 from the molecular ion for levofloxacin (*m/z* = 362), which is common for fluoroquinolones [19], was monitored in the 362 > 318 transition for this analyte. For sertraline, the ion at *m/z* = 277 was formed after the neutral loss of NH_2CH_3 from the parent ion with a *m/z* value of 308. The molecular ion of sulfamethoxazole (*m/z* = 254) and the product ion H_2N-C_6H_4-SO_2 (*m/z* = 156), which is a typical fragment from sulfonamides [10], was monitored. The parent–daughter transition of 291 > 123 corresponded to the molecular ion for trimethoprim and the fragment ion obtained after the loss of $(CH_3O)_3$-C_6H_5 from the parent ion.

Laboratory and field persistence

The experimental k_{obs} for the pharmaceuticals in the laboratory persistence study only were used to assess whether the rates of degradation of the parent pharmaceuticals were different in natural pond water and autoclaved pond water. Because the Pyrex bottles used in this study did not contain sediment, any loss of the pharmaceuticals must be by chemical means. Over the duration of the 30-d laboratory persistence study, none of the pharmaceuticals significantly degraded in the sterile or nonsterile pond water solutions that were stored in the dark. Thus, hydrolytic or microbial degradation were not significant loss processes. Degradation of the compounds was observed in the samples that were light exposed, which suggests sunlight appeared to be important in limiting the persistence of these pharmaceuticals in surface waters by direct or indirect means (illustrative examples using acetaminophen, trimethoprim, and carbamazepine are shown in Fig. 3a–c). The rates of loss of the pharmaceuticals in sunlit autoclaved pond water and natural pond water also did not appear to vary greatly (see Fig. 3). A comparison of the mean half-life in sterile and nonsterile pond water using a standard two-tailed *t* test assuming equal variance (α = 0.05, H_0 = no difference), demonstrated that there was no significant difference between the two means (data not shown). The data for all drugs from the roof experiment showed *p*-values well above 0.05, which supported H_0 and thus the null hypothesis of no difference was accepted. Therefore, the biodegradation of the compounds of interest was not an important loss process in sunlit surface water over the course of the study.

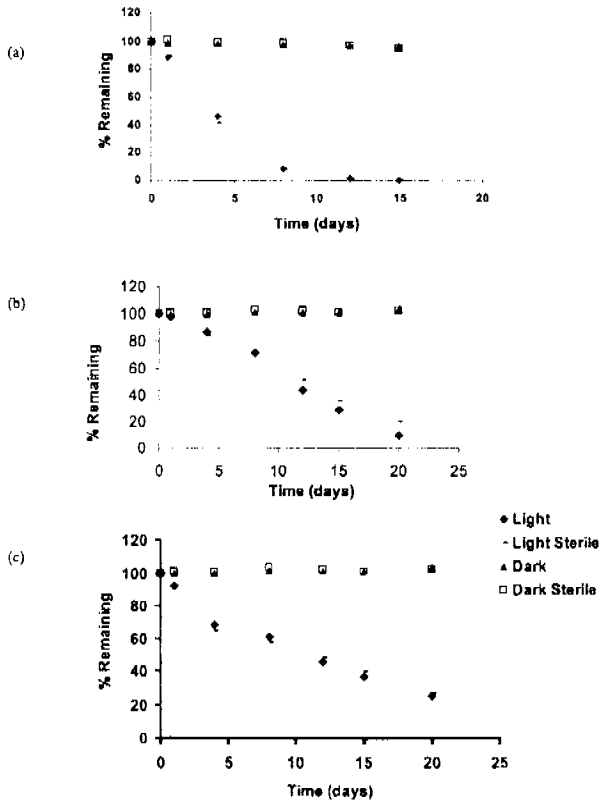

Fig. 3. Aquatic persistence of (**a**) acetaminophen, (**b**) trimethoprim, and (**c**) carbamazepine in dark- and light-exposed sterile and nonsterile pond solutions.

The majority of the pharmaceuticals absorb little or no radiation above 290 nm as determined from their respective UV-vis spectra, which suggests indirect photoreactions could be contributing to the overall fate of these chemicals. The reactivity of the hydroxyl radical ($^{\cdot}OH$) toward pesticides, which are considered more traditional organic pollutants in aquatic systems, has been demonstrated by numerous studies [20,21], and thus indirect photolysis reactions involving $^{\cdot}OH$ could potentially contribute to the degradation of pharmaceuticals in surface waters containing natural $^{\cdot}OH$ producers. Second-order rate constants for reactions of selected pharmaceuticals, including carbamazepine and sulfamethoxazole, with $^{\cdot}OH$ have been published recently; these values range from 3.3 to 9.8 × 10^9 $M^{-1}s^{-1}$ [22], which indicates these compounds could readily react with $^{\cdot}OH$ to limit their persistence in natural waters. Previous studies have shown the carbonate radical ($CO_3^{\cdot-}$) generated from the reaction between $^{\cdot}OH$ and carbonate/bicarbonate species in sunlit surface field waters are important in limiting the persistence of sulfur-containing compounds [23,24]. Thus, $CO_3^{\cdot-}$ may have had a role the transformation of sulfamethoxazole in the microcosms. Ciprofloxacin, which structurally is similar to levofloxacin, has been shown to be susceptible to indirect photoreactions [25], which suggests that levofloxacin could also undergo indirect photolysis.

The control pond water was analyzed to determine the concentration of dissolved organic matter (DOM), a known producer of radicals required for indirect photolysis reactions. Using a total organic carbon analyzer, the concentration of

Table 4. Average half-life of pharmaceuticals at each treatment level (n = 3) and the overall mean half-life of each compound in the microcosms (n = 12)

Compound[a]	UHT[b] $t_{1/2}$ (days)	HT[c] $t_{1/2}$ (days)	MT[d] $t_{1/2}$ (days)	LT[e] $t_{1/2}$ (days)	Mean $t_{1/2}$ (days)
ACT	1.1 ± 0.5	0.7 ± 0.2	0.9 ± 0.1	1.0 ± 0.3	0.9 ± 0.2
ATR	6.9 ± 2.0	6.4 ± 2.3	6.4 ± 0.9	6.5 ± 0.4	6.6 ± 0.2
CAF	1.7 ± 0.2	1.7 ± 0.6	0.9 ± 0.1	1.5 ± 0.2	1.5 ± 0.4
CAR	90.1 ± 4.0	69.7 ± 9.3	76.1 ± 2.5	92.6 ± 3.9	82 ± 11
LEVO	4.9 ± 0.8	4.9 ± 0.9	5.1 ± 1.5	5.3 ± 0.2	5.0 ± 0.1
SRT	6.3 ± 1.3	6.0 ± 1.6	6.5 ± 5.4	6.2 ± 1.2	6.3 ± 0.2
SMX	18.7 ± 6.7	19.4 ± 3.1	17.5 ± 1.3	20.3 ± 2.9	19.0 ± 1.2
TRPRM	ND[f]	ND	5.7 ± 4.2	5.6 ± 1.8	5.7 ± 0.1

[a] ACT = acetaminophen; ATR = atorvastatin; CAF = caffeine; CAR = carbamazepinel; LEVO = levofloxacin; SRT = sertraline; SMX = sulfamethoxazole; TRPRM = trimethoprim.
[b] UHT = ultra high treatment.
[c] HT = high treatment.
[d] MT = medium treatment.
[e] LT = low treatment.
[f] ND = not determined.

DOM was determined to be 2.27 ± 0.02 mg C/L. Although nitrate also is considered to be a primary producer of radicals, namely $^{\bullet}OH$, its concentrations were not measured because the pond water had been in storage at 4°C for longer than the recommended holding time of water used for nitrate analysis. The mean alkalinity and pH for all ponds were determined to be 194.4 ± 11.5 meq/L and 7.8 ± 0.2, respectively. From these values the concentrations of bicarbonate and carbonate ions were calculated to be 1.94×10^{-4} and 5.49×10^{-7} M, respectively. The average solar irradiance that would be expected over the microcosms and over the roof of Lash Miller Chemical Laboratories on a clear summer day at 40°N latitude is 2.69×10^{-4} millieinsteins.cm^{-2}·day^{-1} at 300 nm [26].

In addition to being a producer of $^{\bullet}OH$, DOM also is thought to be a light attenuator and its presence may reduce the photon fluence rate, which in turn could slow the rate of direct and indirect photochemical reactions. Using the control pond water, a screening factor at 300 nm, S(λ), was calculated to determine the transparency of the surface water of the ponds and to assess the amount of light penetration in the microcosms. An explanation for the calculation for screening factors has been described elsewhere [27]. A screening factor was calculated to be 0.93 at a depth of 5 cm in the microcosms. Thus, the average photon fluence rate with 2.27 ± 0.02 mg C/L present in the matrix was 93% of the near-surface photon fluence rate, and rates of degradation may be reduced.

To evaluate the field persistence of the pharmaceuticals, all the microcosms were treated with the appropriate concentrations of the pharmaceutical mixture and samples were drawn at predetermined posttreatment time points and analyzed for the amount of parent compound remaining. The average half-lives determined from average k_{obs} values for each treatment level and the overall mean half-life for each compound are listed in Table 4. Acetaminophen and caffeine were the least persistent, with average half-lives of about 1 d, followed by levofloxacin, trimethoprim, sertraline, and atorvastatin, which had half-lives ranging from 5 to 7 d. The most persistent compounds were sulfamethoxazole and carbamazepine, which had mean half-lives of 19 and 82 d, respectively, in the microcosms. Carbamazepine also was suggested to be fairly persistent in surface waters in a recent study where the occurrence and fate of this pharmaceutical and others were examined [28,29]. Observed half-lives did not vary significantly between treatment levels indicating degradation was not concentration dependent. Because the loss of these pharmaceuticals was not observed in dark control experiments performed in laboratory persistence studies, this suggests that sunlight was important in limiting the persistence of the pharmaceuticals that had shorter half-lives in the microcosms.

The sorption of fluoroquinolones to clays and sewage sludge [30,31], and the sorption of sulfonamides in soil systems have been considered previously [32], and it is possible that soil sorption could be a loss process for some of the pharmaceuticals in the microcosms that were lined with soil. However, photolysis still could be a significant loss process in surface waters because photodegradation of the pharmaceuticals was observed in the solutions that did not contain any soil used in the ambient photoperiod persistence studies.

Degradation metabolites

Degradation products have been identified in previous degradation studies performed for levofloxacin, sulfamethoxazole, and trimethoprim [9,10,12]. The degradation products of levofloxacin in aqueous solution have been identified as analogues altered at the *N*-methylpiperazine moiety of the compound [9]. The photoisomerization of the isoxazole ring of sulfamethoxazole leads to the formation of its major degradation product 4-amino-*N*-(5-methyl-2-oxazolyl)benzenesulfonamide [10]. Two other primary photoproducts of sulfamethoxazole are formed from the cleavage of the $-SO_2$-NH- linkage into sulfanilic acid and 3-amino-5-methylisoxazole [10]. The main degradation products of trimethoprim that could form under the conditions in the microcosms are produced from the hydrolysis of the amino groups and oxidation of the benzylic methylene function [12]. Hydrolysis of the *p*-methoxy group has been observed under extreme acidic conditions [12] and this photoproduct is not expected to form in the microcosms.

Due to the limited supply of microcosm water samples, these samples could not be analyzed for known photodegradation products. However, a photodegradation study currently is being done for atorvastatin, carbamazepine, levofloxacin, and sulfamethoxazole in pure water. Preliminary results obtained by LC-MS-MS show that the photoisomer of sulfamethoxazole is formed quickly after several minutes of irradiation. This imido tautomeric form of the parent compound is identical in mass and would have the same precursor-product ion transition as sulfamethoxazole. However, the parent and this photoisomer can be fully resolved by chromatography [10]

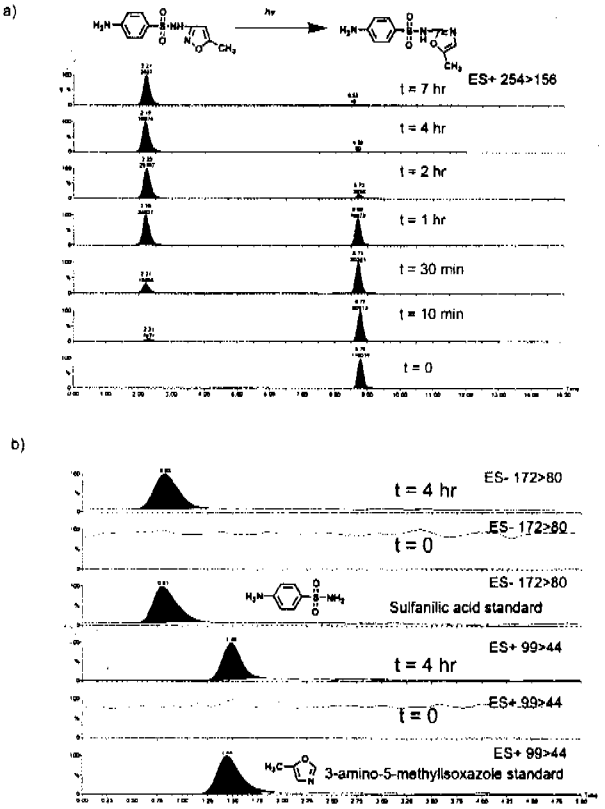

Fig. 4. Liquid chromatography tandem mass chromatograms of (a) sulfamethoxazole the production of its photoisomer (b) sulfanilic acid and 3-methyl-5-aminomethylisoxazole as photoproducts of sulfamethoxazole (ES = electrospray).

as demonstrated by HPLC-UV (data not shown) and LC-MS-MS (see Fig. 4a). An authentic standard of the photoisomer was not available for comparison, but the UV-vis spectra of this photoisomer observed by Zhou et al. [10] was in agreement with that obtained in this study. The production of sulfanilic acid and 3-amino-5-methylisoxazole also were observed and confirmed with standards by LC-MS-MS (see Fig. 4b). The photoisomer and the latter two photoproducts are formed after 10 min of irradiation and their chromatographic areas increased to a maximum after 6 h of radiation, but these compounds did not appear to accumulate and their presence was no longer observed after 24 h of irradiation.

On the basis of the molecular peaks and fragments detected, a structure for one of the observed molecular ions observed in the photolysate of atorvastatin can be suggested. Preliminary results in the photodegradation study of atorvastatin suggest that the parent compound undergoes a photonucleophilic aromatic substitution reaction whereby the F atom is substituted by OH. A parent–daughter transition possibly corresponding to the mass of the defluorinated photoproduct and daughter ions formed by the cleavage of C_6H_5-NCO [M-119] or the cleavage of $C_6H_5NH_2$ [M-93] were observed when an irradiated sample was concentrated by SPE and infused to the mass spectrometer. The [M+H] ions with a *m/z* of 555 and 416, and a [M-H] ion with a *m/z* of 573 also were observed in the infusion of the concentrated photolysate. The 555 *m/z* ion also had a parent–daughter transition corresponding to the cleavage of C_6H_5-NCO and $C_6H_5NH_2$ from the parent ion, while the 416 *m/z* ion had a parent–daughter transition corresponding to the loss of $C_6H_5NH_2$. A loss of $C_4H_8O_3$ from the heptanoic side chain of the molecule corresponded to the 573 > 469 transition. The molecular mass of this particular ion is greater than the parent atorvastatin by 16 amu, which suggests this compound formed is an oxygenated photoproduct. The loss of $C_4H_8O_3$ (M-104) from the 7-carbon side chain also is observed for the parent atorvastatin molecule in negative ionization mode. The structures of these degradation compounds remain unknown, but further MS-MS studies in positive and negative modes using electrospray and atmospheric pressure chemical ionization will be conducted to suggest possible structures. The photoproducts of atorvastatin have MS-MS spectra showed characteristic and structurally informative amide bond fragmentation in the positive mode. A previous study of the metabolism of atorvastatin in mice showed that the major metabolites that retained the amide linkage also consistently lost C_6H_5-NCO [M-119] and $C_6H_5NH_2$ [M-93] as fragment ions in positive mode [33]. The evolution of the photoproducts was analyzed using the parent–daughter transitions mentioned above. Figure 5 shows chromatographic peak area versus irradiation time. The photoproducts were formed within the first 10 min of light absorption and the peak area for the compound corresponding to 557 > 438 reached a maximum after 2 h while that for 555 > 436 and 416 > 323 reached a maximum after approximately 5 h. These photodegradation products did not appear to accumulate and were not observed after 24 h of irradiation.

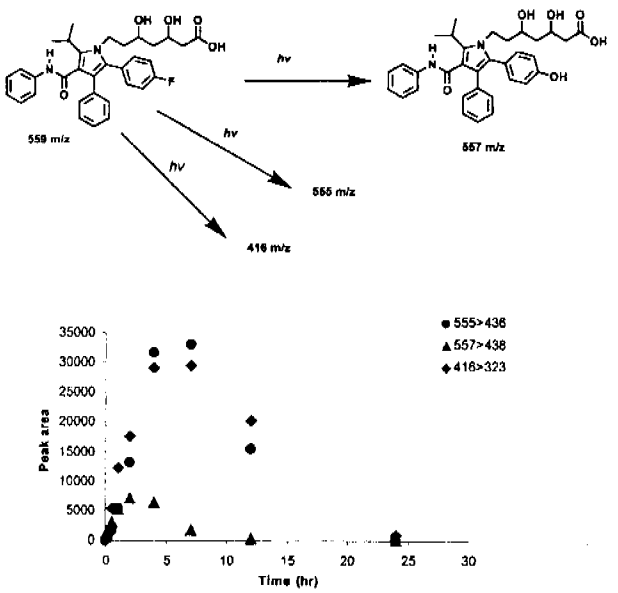

Fig. 5. Concentration profile of unidentified photodegradation product and structure of suggested photoproduct of atorvastatin over the course of 24 h of irradiation.

The production of several known photoproducts of levofloxacin was suggested following LC-MS-MS analysis of irradiated solutions. These photodegradation products of levofloxacin, which are analogues altered at the *N*-methylpiperazine moiety of the parent compound, previously were observed and characterized by fast atom bombardment-MS and electron impact-MS by Yoshida et al. [9]. The production of the photoproducts of levofloxacin observed in this study reached a maximum by 2 h, after which their concentrations decreased (see Fig. 6).

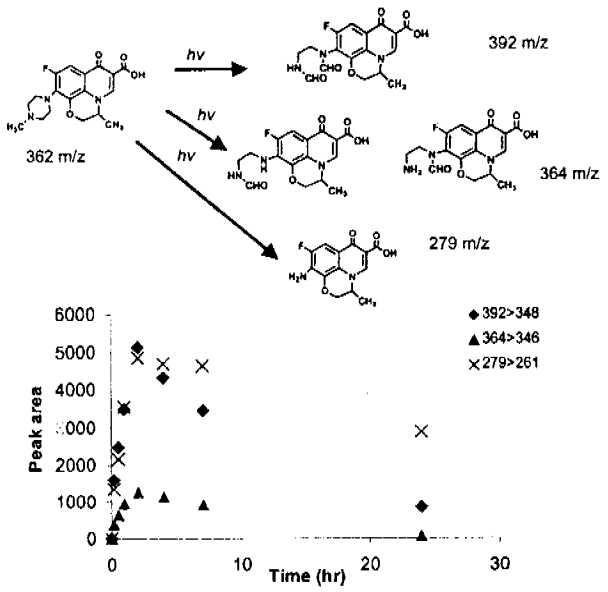

Fig. 6. Structures of photoproducts of levofloxacin and their concentration profile over the course of 24 h of irradiation.

Carbamazepine was the compound most resistant to photodegradation with a half-life of approximately 1 d in the sunlight simulator. This compound has been reported to degrade and be transformed under irradiation into the 10,11-epoxide (252 g/mol) [34]. The LC-MS-MS analysis in single ion monitoring mode of sample aliquots taken after 24 h of irradiation suggest that the epoxide is formed. A parent ion with a *m/z* of 194 also was observed in the photolysates; this mass corresponded to iminostilbene as a possible degradation product that could form after the loss of HN=C=O from carbamazepine. A daughter ion scan was performed on the ion observed in the photolysate and the resulting fragmentation pattern resembled that of a purchased standard of iminostilbene. However, a previous study using GC-MS to quantify carbamazepine in surface waters suggests that this compound is degraded thermally to iminostilbene [35]. The molecular ions for both carbamazepine and iminostilbene were observed in an infused standard containing only carbamazepine to the LC-MS-MS, which suggested this compound could undergo thermal degradation in the heated desolvation chamber. Lowering the desolvation temperature and increasing the desolvation gas flow rate reduced the degree of degradation, but the intensity of the molecular ion of carbamazepine was reduced greatly. However, it is unlikely that iminostilbene is a photodegradation product of carbamazepine because the chromatographed peak corresponding to the parent–daughter transition for iminostilbene did not increase over the course of a carbamazepine photodegradation experiment. Furthermore, the presence of iminostilbene in these photolysates was not observed using HPLC-UV, a technique where the potential for thermal decomposition of carbamazepine is less likely.

The observed photoproducts of atorvastatin, levofloxacin, and sulfamethoxazole still appear to be photoreactive in sunlit surface waters as their presence was not observed after 24 h of irradiation in the sunlight simulator. However, if the parent compounds are infused continually to the aquatic environment, the parent compound and the photoproducts have the potential to be present constantly. Authentic standards for the observed photodegradation products of levofloxacin and atorvastatin in this study were not available for comparison of retention time and mass spectral data. A mass balance for parents and observed photoproducts has not been determined because there are likely to be many more photometabolites, some of which could be more persistent than the parent pharmaceutical. It is important to account for the total effect of parent compounds and their potential degradation products to understand completely the environmental chemistry of pharmaceuticals and other organic contaminants in the environment. The degradation compounds produced as a result of photolysis should be accounted for, both for mass balance and in examining their ecological and toxicological effect. An investigation into the contribution of indirect photolysis to the overall photolytic fate of these chemicals and the degradation products formed will be presented and discussed in a future paper.

CONCLUSION

Fast, simple, and robust HPLC-UV and LC-MS-MS methods for analyzing a mixture of eight pharmaceuticals were developed to monitor their concentration in outdoor microcosms. Photolysis of the pharmaceuticals studied in sunlit surface water was an important loss process in limiting their persistence in the aquatic environment. Biodegradation did not appear to be important in the loss of these compounds in the microcosms. The photodegradation products of atorvastatin, levofloxacin, and sulfamethoxazole appear to be photoreactive and are subject to further degradation in sunlit surface waters.

Acknowledgement—The authors acknowledge Stacey Smith for the collection of microcosm water samples and for the maintenance of the microcosms. The authors would like to thank the National Science and Engineering Research Council of Canada and Canadian Network of Toxicology Centres for research funding, and Perkin Elmer for the creation of the ANALEST facility (University of Toronto, Toronto, ON, Canada).

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