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Stability and Removal of Atorvastatin, Rosuvastatin and Simvastatin from Wastewater

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Abstract

Atorvastatin (ATO), Rosuvastatin (RST) and Simvastatin (SIM) are commonly used drugs that belong to the statin family (lowering human blood cholesterol levels) and have been detected as contaminants in natural waters. Stability and removal of ATO, RST and SIM from spiked
wastewater produced at Al-Quds University Campus were investigated. All three statins were found to undergo degradation in wastewater (activated sludge). The degradation reactions of the three drugs in wastewater at room temperature follow first order kinetics with rate constants of 2.2 x 10^{-7} s^{-1} (ATO), 1.8 x 10^{-7} s^{-1} (RST) and 1.8 x 10^{-6} s^{-1} (SIM), which are larger than those obtained in pure water under the same conditions, 1.9x 10^{-8} s^{-1} (ATO), 2.2x 10^{-8} s^{-1} (RST) and 6.2 x 10^{-7} s^{-1} (SIM). Degradation products were identified by LC-MS and LC/MS/MS. The overall performance of the wastewater treatment plant (WWTP) installed in Al-Quds University Campus towards the removal of these drugs was assessed showing that more than 90% of spiked ATO, RST and SIM were removed. In order to evaluate the efficiency of alternative removal methods to replace ultra-filtration membranes, adsorption isotherms for the three statins were investigated using both activated carbon and clay-micelle complex as adsorbents. The batch adsorption isotherms for the three statins were found to fit Langmuir equation, with larger number of adsorption sites and binding affinity for micelle-clay composite compared to activated carbon and filtration experiments of the three statins and their corresponding metabolites demonstrated a more efficient removal by micelle-clay filters.

Keywords: Statins - Wastewater treatment - Stability in sludge - HF-membranes - Activated carbon - Micelle–clay complex.

Nomenclature:

ATO: Atorvastatin
RST: Rosuvastatin
SIM: Simvastatin
OWCs: Organic wastewater compounds
PhACs: Pharmaceutically active compounds
LDL: Low-density lipoprotein
HMG-CoA: 3-Hydroxy-3-methylglutaryl coenzyme A
CMC: Critical micelle concentration
FAC: Fine powder activated charcoal
GAC: Granular activated charcoal
HF: Hollow fiber UF membrane
IRMPD: Infrared multiphoton dissociation
LC/FT-ICR MS: Liquid chromatography/Fourier-transform ion cyclotron resonance mass spectrometry
MC: Micelle-clay complex
ODTMA: Octadecyltrimethylammonium
RO: Reverse osmosis
SW: Spiral wound UF membrane
UF: Ultra-filtration
WWTP: Wastewater treatment plant

1. Introduction

Widespread development in watershed recharge areas increases the likelihood of contamination in surface water and groundwater resources by wastewater effluents.[1] These effluents are contaminated with variety of organic wastewater compounds (OWCs) such as excreted hormones and pharmaceuticals, detergent components, and disposed household and personal care products. These contaminants attracted special concern because of their ability to interfere with the function of natural hormones in both aquatic organisms and humans.[2-5] Many of these compounds have been widely documented in surface waters receiving discharge from wastewater treatment plants.[6-12]

In recent years, the environmental occurrence of pharmaceutically active compounds (PhACs), human and veterinary medication, has been a source of growing concern [13]. It has been shown that they can adversely affect both aquatic and non-aquatic organisms and thus the ecosystem [13]. In most cases current water and wastewater treatment systems do not completely remove PhACs [14, 17].

Statins are a group of pharmaceuticals used for lowering cholesterol levels in the blood. They are commonly applied for the reduction of cardiovascular-related morbidity and mortality in patients with or at risk of coronary heart diseases. In the long and complex biosynthesis of cholesterol in humans, the first and rate-determining step is the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which catalyzes the conversion of HMG-CoA to mevalonic acid.[18,19]
Clinical studies have demonstrated that statins are potent and competitive inhibitors of HMG-CoA reductase for cholesterol synthesis, thereby limiting the hepatic production of low-density lipoprotein (LDL).\[20\] The fully synthetic ATO, RST and SIM are presently the three most popular, second generation HMG-CoA reductase inhibitors in the statin family.\[18\]

Atorvastatin (ATO, structure 1, Figure 1), [(3R,5R)-7-[2-(4-Fluoro-phenyl)-4-(phenylcarbamoyl)-5-isopropyl-3-phenyl-pyrrol-1-yl]-3,5-dihydroxy-heptanoic acid, is one of the most prescribed drugs.\[20\] Recently it has been observed that ATO, like other statins, can be efficient against Alzheimer’s disease.\[20\] Less than 5% of a dose of ATO is recovered in urine following oral administration. The presence of ATO in sewage effluents and surface waters has been observed in concentration levels of µg L\(^{-1}\).\[21\]

Pharmacokinetic studies have shown that, after oral administration, ATO was rapidly metabolized into two active hydroxy metabolites (2-hydroxy-ATO and 4-hydroxy-ATO) and three inactive lactone metabolites.\[22,23\] However, the lactones were unstable as they hydrolyzed readily to their original acid forms. In contrast, RST was found not to be extensively metabolized, as 77% of it was excreted unchanged.\[24\] Approximately 90% of the non-metabolized RST was recovered in feces, with the remaining 10% in urine. Two minor metabolites, the RST-5S-lactone (RSTL) and the N-desmethyl RST, were identified by LC-NMR and LC–MS/MS in the same study.\[24\]

Atorvastatin was found to undergo a self-sensitized photo oxygenation by sunlight in water \[20\](Cermola et al. 2006). The main photoproducts, isolated by chromatographic techniques, were identified by spectroscopic means. They present a lactam ring arising from an oxidation of pyrrole ring and an alkyl/aryl shift. A mechanism involving singlet oxygen addition and an epoxide intermediate was suggested.\[25\]

Rosuvastatin, bis [(E)-7-[4-(4-fluorophenyl)-6-isopropyl-2-[methyl (methylsulfonyl) amino] pyrimidin-5-yl] (3R, 5S)-3,5-dihydroxyhept-6-enoic acid] (RST, structure 2, Figure 1), decreases the production of LDL cholesterol by blocking the action of the enzyme HMG-CoA reductase in the liver which is responsible for its production. This decreases the amount of cholesterol in the liver cells, which causes them to take up LDL cholesterol from the blood. The
decreased cholesterol production and increased removal of LDL cholesterol from the blood ultimately results in lowered blood cholesterol levels.[26]

In the pharmacokinetic study of RST, RSTL (structure 4, Figure 1) was identified as one of the minor metabolites.[24]

Simvastatin, is (+) (1S,3R,7S,8S,8αR)-1,2,3,7,8,8α-hexa-hydro-3,7-dimethyl-8-[2-[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl]-1-naphthyl-2,2-dimethyl butanoate (SIM, structure 3, Figure 1), is derived synthetically from a fermentation product of Aspergillus stercoratus.[27]

After oral ingestion, Simvastatin, which is an inactive lactone, is hydrolyzed to the corresponding β-hydroxyacid form. The latter is a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, an essential enzyme involved in the in vivo synthesis of cholesterol.[28, 29]

The goals of this study were (i) to explore the efficiency of the integrated advanced wastewater treatment technology towards the removal of Atorvastatin, Rosuvastatin and Simvastatin and their metabolites from spiked wastewater samples; (ii) to evaluate the degradation kinetics of the above mentioned compounds in water and sludge, and identify the biodegradation products; (iii) to determine the ability of micelle-clay composite and activated carbon towards removal of these drugs by adsorption from aqueous solutions. Furthermore, adsorption kinetics and adsorption isotherms are evaluated and fitted to Langmuir equations. The micelle-clay composite which was used in this study is positively charged, has large surface area and includes large hydrophobic domains. Micelle-clay composites have already been proven useful in the removal of about 20 neutral and anionic pollutants.[30]

2. Experimental

2.1 Materials and methods

2.1.1 Materials
All purchased chemicals were of analytical grade. The clay used was Wyoming Na-montmorillonite SWY-2 clay; obtained from the Source Clays Registry (Clay Mineral Society, Colombia, MO). Quartz sand (grain size 0.8—1.2 mm) was obtained from Negev industrial minerals (Israel). Octadecyltrimethylammonium (ODTMA, structure 5 in Figure 1) bromide was obtained from Sigma Aldrich. Pure ATO, RST and SIM (compounds 1, 2 and 3 in Figure 1, respectively) were obtained from Birzeit Pharmaceutical Company (Palestine) as standard products with 99% purity, and were used all as received. Fine powder activated charcoal (FAC) with particle size ≤ 60 µm, and granular activated charcoal (GAC) with particle size ≤ 700 µm were obtained from Sigma (Sigma Chemical Company, USA). The powder was used for batch adsorption experiments whereas the granules were used in column experiments. Magnesium sulfate anhydrous, acetonitrile as well as methanol and water for analysis (HPLC grade) were purchased from Sigma Aldrich (Munich, Germany). High purity diethyl ether (> 99%) was purchased from Biolab (Israel).

For sample enrichment and purification SPE 1g C-18 6 mL disposable cartridges (Waters, Milford, MA, USA) were used.

Equipment: Samples were shaken using Big Bill, (Banstaed/ Themolyne, USA). The disappearance of ATO, RST and SIM was determined by using a high pressure liquid chromatography system model 2695 HPLC (Waters, USA), equipped with a Waters 2996 Photodiode array. Data acquisition and control were carried out using Empower™ software (Waters, USA). Analytes were separated on a 4.6 mm x150 mm C18 XBridge® column (5 µm particle size) used in conjunction with a 4.6 mm, 20 µm, XBridge® C18 guard column.

HPLC conditions: mixture of 1% H₃PO₄: acetonitrile (1:1; v/v) as mobile phase; flow rate of 1.5 mL min⁻¹; UV detection at a wavelength of 238 nm. Acrodisc® syringe filters with GHP membrane (hydrophilic polypropylene 0.45µm porosity) from Waters were always used for all analytical filtration requirements [26](BP-2007). The identification of ATO, RST and SIM degradation products was performed using a liquid chromatography system coupled to a hybrid linear quadrupole ion trap (LTQ) – Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

Analyses were performed by using the same column in a isocratic mode with two solutions, solution A: water with formic acid (0.1% v/v) and solution B: acetonitrile (100% v/v) [ 35:65] at ambient temperature at a flow rate of 1.0 mL/min, which was split 4:1 after the analytical column
to allow 200 µl min⁻¹ to enter the ESI source. Negative and positive ion ESI-MS was used for the
detection of compounds of interest. Mass spectrometric conditions were optimized by direct
infusion of standard solutions. The instrument was tuned to facilitate the ionization process and
to achieve the highest sensitivity. The MS detector was tuned whenever the solvent flow rate
conditions were changed, and the electrospray voltage, heated capillary temperature and voltage,
tube lens voltage, sheath gas flow rate and auxiliary gas flow rate were optimized until the ion
transmission was maximized. Full-scan experiments were performed in the ICR trapping cell in
the range m/z 50–1000. Mass-to-charge ratio signals (m/z) were acquired as profile data at a
resolution of 100,000 (FWHM) at m/z 400. Data acquisition and analyses were accomplished
using the Xcalibur software package (version 2.0 SR1 Thermo Electron). The simplest method to
identify analytes by eXtracted Ion Chromatograms (XICs) was used. XIC collects ion intensities
falling within a given mass-to-charge-ratio window and appears to be the method of choice for
mass analysis leading to very simple chromatograms. The reduction of interferences in the XICs
significantly facilitates the identification of putative metabolites. Data were collected in full MS
scan mode and processed post-acquisition, to reconstruct the elution profile for the ions of
interest, with a given m/z value and tolerance. The chromatographic raw data were imported,
elaborated and plotted by SigmaPlot 10.0 (Systat Software, Inc., London, UK).

The advanced wastewater treatment plant employed in this study is located at Al-Quds
University-Palestine and was described in detail elsewhere.[31] Normally, the effluent from this
plant is recycled for the irrigation of plants cropped in the fields of the University Campus.

2.2 Methods

2.2.1 Characterization of wastewater used.

The chemical and biological quality of wastewater before and after treatment was characterized
according to American Public Health Association procedures [32, 33] by performing
measurements listed in Table S1 (see Supplementary data).

2.2.2 Efficiency of WWTP for ATO, RST and SIM removal.
The efficiency of different treatment units was determined by spiking separately the secondary effluent with 1.0 mg L\(^{-1}\) of ATO, RST and SIM in the activated sludge reservoir (1000 L). Samples were collected from different locations of the WWTP as depicted in Figure S1, Supplementary data. SPE-C18 disposable cartridges were used to pre-concentrate 10 mL of each sample by adsorption of analytes. A part (20 µL) of the methanolic solution eluted from SPE cartridge was injected into the HPLC, and analyzed using the same conditions for the determination of ATO, RST and SIM. Recovery tests were performed using triplicate solutions of the three substances, and values ranging from 98% to 102% were obtained.

2.2.3 Stability of ATO, RST and SIM.

Stability studies of ATO, RST and SIM were performed using 100 mg L\(^{-1}\) solutions in pure water, or activated sludge taken from the secondary treatment stage of the WWTP installed at Al-Quds University. At specific time intervals (0 to 35 days) samples were collected from the solutions (maintained under continuous orbital shaking), filtered, and analyzed by HPLC. The degradation by-products of ATO, RST and SIM were quantified using liquid chromatography/Fourier-transform ion cyclotron resonance/mass spectrometry (LC/FT-ICR MS).

2.2.4 Micelle-clay complex preparation.

The ODMTA micelle-clay complex was prepared by mixing a smectitic clay mineral (montmorillonite) with the cationic surfactant octadecyltrimethylammonium (as bromide salt) at multiples of 10 g L\(^{-1}\) (clay) and 12 mM (ODTMA, 5 in Figure 1) as described elsewhere (Karaman et al. 2012). The obtained complex, which has net positive charge and includes hydrophobic regions, is capable of efficiently binding neutral and negatively charged organic molecules [34-37] (Polubesova et al. 2005; Polubesova et al. 2006; Zadaka et al. 2007; Mishael et al. 2002).

2.2.5 Batch adsorption experiments.
Batch adsorption experiments were carried out on ATO, RST and SIM at different concentrations. Experiments were performed in 250 mL Erlenmeyer flasks containing 200 mg of either micelle-clay complex or fine powder activated charcoal (FAC). 100 mL of each drug solutions having known initial concentration were then introduced into each flask. The flasks were shaken in an oscillating shaker for three hours at room temperature, and then 2.0 mL portions were filtered using 0.45 μm filters. Equilibrium concentrations of ATO, RST and SIM were then obtained by HPLC, using the conditions reported above. The retention times of ATO, RST and SIM were 6.6, 3.7 and 3.2 minutes, respectively.

2.2.6 Column filtration experiments.

Column filtering experiments were performed using 50/1 (w/w) mixtures of quartz sand and either ODTMA-clay complex, or granular activated charcoal (GAC), 20 cm layered in borosilicate columns of 25 cm length and 5 cm internal diameter. Each column contained 13 g of complex, or GAC. The bottom of the column was covered with 3 cm layer of quartz sand. Quartz sand was thoroughly washed by distilled water and dried at 105 °C for 24 hours before its use. Solutions in pure water (1 L each) containing different ATO, RST and SIM concentrations (0.01, 1, 10, and 100 mg L⁻¹) were passed through either micelle-clay or GAC columns (one column for each solution). In all cases the flow rate was 2.0 mL min⁻¹. Eluted fractions were collected in all column experiments and analyzed.

All experiments reported were performed in three replicates and average values and standard deviations were calculated.

3. Results and discussion

3.1 Calibration curves

Linearity of the proposed analytical method was verified by analyzing standard solutions in the range of 0.1 - 100 mg L⁻¹ for ATO, RST and SIM in pure water. The calibration curves were obtained using HPLC method with calculated regression coefficient ranging from 0.9998 to 0.9999. The reproducibility of triplicate subsequent injections ranged from 98.4% to 99.6%, depending on the sample concentration and type of analyte. The reproducibility of morning/evening injections on the basis of 6-hours elapsed time ranged from 97.5% and 98.0%,
and was also affected by the concentration and type of analyte. Correction coefficients were used for experimental samples.

Calibration curves and reproducibility trials were repeated preparing new calibration solutions by using wastewater taken from the activated sludge reservoir of Al-Quds WWTP. Results suffered of a minor accuracy due to the variability of recovery percentages. Anyway, the determination coefficients of calibration curves were 0.9997 for ATO, 0.9995 for RST and 0.9999 for SIM. The limit of detection, based on a signal/noise of 3, was 0.03 mg L⁻¹ for ATO and RST, and 0.02 mg L⁻¹ for SIM. The limit of quantifications, based on a signal/noise of 10, was 0.08 mg L⁻¹, 0.08 mg L⁻¹ and 0.06 mg L⁻¹ for ATO, RST and SIM, respectively.

### 3.2 Wastewater characteristics

Table S2 (see Supplementary data) summarizes the chemical, physical and biological characteristics of wastewater sampled from the activated sludge reservoir of Al-Quds WWTP. Table S2 reveals that the wastewater contained high amounts of suspended solids and large populations of bacteria, which are responsible for fouling phenomena affecting ultra-filtration and reverse osmosis membranes. Moreover, high values of electrical conductivity and total dissolved solids, are typical for municipal wastewaters, and should be reduced if WWTP effluents are re-used for crop irrigation purposes.

### 3.3 Efficiency of WWTP for ATO, RST and SIM removal

The efficiency of WWTP at Al-Quds University for the removal of ATO, RST and SIM was studied. The activated sludge reservoir (site 1 in Figure S1; Table S2) was separately spiked with ATO, RST or SIM at concentration of 1.0 mg L⁻¹, which is an amount close to environmental values reported in the literature.[21,38] Samples were taken from different collecting sites of WWTP as described in Figure S1. Analytical results of water effluent from the hollow fiber ultra-filtration membrane (UF-HF) indicated that ATO, RST and SIM were about 84.6%, 69.2% and 73.6% removed at this stage, respectively, whereas about 100% of ATO, RST and 90.7% of SIM were removed after passing the spiral wound (UF-SW) membrane (Table 1). Besides, RST was completely removed in the effluent from GAC filter. However, it should be outlined that the concentration of ATO, RST and SIM influent in the treatment units were diminishing along their
sequence. This resulted in 100% removal by GAC filter, whose influent water contained only 0.067 mg L\(^{-1}\) of RST, on average, after the passage through the UF filters. This finding made unnecessary the use of reverse osmosis for any further purification. Nevertheless, the advanced technology adopted in the WWTP of Al-Quds University did not overcome a problem common to all plants: the production of brine, in which a large portion of the contaminants ends up being concentrated there. For this reason additional methods of water filtration and purification were experimented.

3.4 Stability of ATO, RST and SIM in pure water and in sludge

Since many pharmaceuticals might undergo degradation upon their standing in aqueous medium and sludge environment [38-39] (Bendz et al. 2005; Jones et al. 2005), kinetics studies on degradation of Atorvastatin, Rosuvastatin and Simvastatin in pure water and in activated sludge conditions have been undertaken. Table 2 summarizes the hydrolysis results of the drugs in activated sludge and pure water at room temperature. For brevity we show the detailed results only for Atorvastatin (A) (Fig. 2) and Simvastatin (B) (Fig. 2). The accelerated degradation of the statin drugs in sludge is expected to occur by the activity of bacteria attached to sites in the sludge. Hence the process involves first sorption of the statin molecules by the sludge and then degradation by the bacteria. Consequently, even if the degradation by the bacteria is a first order reaction, the overall reaction may deviate from a first order. However, in our case, where the rate of degradation is slow relative to the adsorption process, the overall rate of the degradation can appear as first order. Table 2 and Figure 2 demonstrates that the assumption of a first order rate of degradation is largely justified.”

Please Insert Figure 2 Here
The results in Table 2 indicate that the rates of degradation for ATO, RST, and SIM in sludge are about 12-, 8- and 3-fold faster than in pure water, respectively. The rate of degradation of SIM in sludge is about an order of magnitude faster when compared with ATO and RST, where the ratio is about 30-fold in water. The higher rate of SIM degradation compared to ATO and RST stems from the fact that SIM exists as a lactone moiety which is readily hydrolyzed to the corresponding carboxylic acid form in the presence of acid or base catalysis, whereas ATO and RST exist in the free carboxylic acid forms.

The accelerated degradation in sludge compared to that in pure water can be attributed to bioactivity of the activated sludge. The morphological characterization of bacterial community in Al-Quds activated sludge allowed to identify many bacterial species: *Escherichia coli*, *Enterobacter sakazakii*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Enterobacter cloacae*, *Enterobacter amnigenus*, *Enterobacter aerogenes*, *Salmonella spp.*, and *Serratia liquefaciens*. Further challenge will be the isolation of strains constituting the bacterial colonies, aiming at the identification of the more active strains capable of utilizing the pharmaceutical molecules as energy source.

Please Insert Table 2 Here

Literature survey on the stability and degradation of SIM indicates that the drug can undergo metabolic degradation in humans.[40] Upon incubation of SIM with liver microsomal preparations from human donors, four major metabolic products were formed (3*-hydroxy SIM, 6*-exomethylene SIM, 3*5*-dihydrodiol SIM, and the active hydroxy acid, SIMA), together with several minor unidentified metabolites.[40] Based on four different in vitro approaches, namely 1) correlation analysis, 2) chemical inhibition, 3) immune inhibition, and 4) metabolism by recombinant human P450, it is concluded that CYP3A is the major enzyme subfamily responsible for the metabolism of SIM by human liver microsomes.[40] Similar results were obtained by Robert et al. (2003)[41], who showed that CYP3A4 was the main enzyme involved in ATO metabolism resulting in the major metabolite ortho-hydroxy ATO and in active metabolites ATO lactone and ortho-hydroxy ATO lactone (Fig. 3). These results are in agreement with a study by Liu et al. (2008)[42] who showed that ATO is administered in its calcium salt active form and is converted into active ATO acid, which is then metabolized into
two other active metabolites, para-hydroxy ATO (p-ATO), and ortho-hydroxy ATO (o-ATO). These three active compounds are subsequently equilibrated with their corresponding lactone forms at the ratio of approximately 1:1 (Liu et al. 2008)[42]. Jani et al. (2010)[43] found that ATO in plasma can be metabolized by CYP3A4 to two hydroxylated metabolites, o- hydroxylatorvastatin and p-hydroxyatorvastatin (Fig. 3).

Please Insert Figure 3 Here

RST is a new generation of HMG-CoA reductase inhibitor, which exhibits some unique pharmacologic and pharmacokinetic properties. It has low extra hepatic tissue penetration, low potential for CYP3A4 interaction and substantial LDL-C lowering capacity and therefore has distinct advantages.[44] Metabolism of RST by cytochrome p450 (CYP) appears to be minimal and is principally mediated by the 2C9 enzyme, with little involvement of 3A4; this finding is consistent with the absence of clinically significant pharmacokinetic drug-drug interactions between RST and other drugs known to inhibit CYP enzymes.[45] The major metabolite of RST is N-desmethyl RST (active) via CYP450 2C9. Greater than 90% of activity is due to N-desmethyl RST. Clearance is not significantly dependent on CYP450 3A4.[44] Monitoring the derivative substances arising from the degradation of ATO, RST and SIM in the activated sludge indicated that ATO underwent degradation to five by-products, whereas RST and SIM gave rise to only one derivative, respectively, as identified by mass spectrometric analysis. Extracted ion chromatograms (XICs) of the 35 days biodegraded sample are shown in Figure 4. The benefit of using very selective extracted ion chromatograms by FTICR MS, generated with a tight mass-to-charge ratio window of +0.0010 units around each selected protonated or deprotonated molecule (i.e. [M+H]+ or [M-H]- ±1.0 mDa), greatly reduced the signal complexity of the total ion current trace (Fig. 4(b)) allowing to completely characterize all degradation products.

Five major biodegradation products for Atorvastatin (Fig.4(a)B, exact [M-H]- m/z 557.24572) were found at retention times 7.5(6), 5.2(7), 4.3(8), 4.5(9) and 6.0(10) minutes, corresponding to compounds with exact [M-H]- m/z ratios 589.23555 (6, Fig.4(a)D), 573.24064 (7, Fig.4(a)C), 547.18860 (8, Fig.4(a)E), 545.17295 (9, Fig.4(a)F), 513.21951(10, Fig.4(a)H). Based on the accurate m/z ratios of deprotonated molecules, their retention times, and relevant literature [41],
we propose for Atorvastatin biodegradation the structures reported in Figure 5. The major metabolites resulted the compound (7), the para-hydroxy Atorvastatin, C\textsubscript{33}H\textsubscript{35}FN\textsubscript{2}O\textsubscript{6}, with exact [M-H]⁻ m/z 573.24064 (error 0.6 ppm). Ortho, para-dihydroxyatorvastatin was, also, recognized at exact deprotonated m/z ratio 589.23555 (error 1.2 ppm). The peaks belong to (8), (9) and (10) can be attributable to compounds obtained from losses of -CH\textsubscript{3}COOH or isopropyl group from other biodegraded, oxidized products.

Rosuvastatin (Fig.4(b)B, exact [M-H]⁻ m/z 480.16101) has one of major biodegradation products at retention time of 5.2(5) minutes (Fig 4(b)E). Based on the accurate m/z ratios 462.15098 and relevant literature [45], we suggest the structure of the metabolite as shown in Figure 1 (structure 4). Other compounds derived from hydroxylation or polihydroxylation (Fig 4(b) C and D) showing an accurate [M-H]⁻ ratio at m/z 496.15614 (exact m/z 496.15592, error 0.4 ppm) and 514.16660 (exact m/z 514.16649, error 0.2 ppm) and attributable at compounds with molecular formula C\textsubscript{22}H\textsubscript{28}FN\textsubscript{3}O\textsubscript{7}S and C\textsubscript{22}H\textsubscript{30}FN\textsubscript{3}O\textsubscript{8}S, respectively, which were also found in the biodegraded solution of Rosuvastatin. For Simvastatin (Fig.4(c), exact [M-H]⁻ m/z 573.24064) one major biodegradation product was formed at retention time of 9.1 (11) minutes. Based on the accurate [M-H]⁻ m/z ratio 435.27560 (error 0.9 ppm) and relevant literature [40], we propose the structure of SIM metabolite as shown in Figure 5 (11). Interestingly, accurate mass data of biodegraded products, as protonated or deprotonated molecules, with a mass error lower than +1.2 ppm was found, indicating a very good mass accuracy.

It is worth noting, that no reports have been published on biodegradation of ATO, RST and SIM in wastewater.

Please Insert Figure 4 Here

Please Insert Figure 5 Here

3.5 Adsorption isotherms
The adsorption of ATO, RST and SIM at several initial concentrations on micelle-clay complex and activated charcoal was investigated. Equilibrium relationships between adsorbent and adsorbate can be described by Langmuir adsorption isotherm [46](Dakiky et al. 2002), represented by equation (1):

$$\frac{C_e}{Q_e} = \frac{1}{KQ_{\text{max}}} + \frac{C_e}{Q_{\text{max}}}$$ \hspace{1cm} (1)

where $C_e$ (mg L$^{-1}$) is the equilibrium concentration of the drug in the solution, $Q_e$ (mg g$^{-1}$) is the equilibrium mass of adsorbed drug per gram of complex or activated charcoal, $K$ (L mg$^{-1}$) is the Langmuir binding constant, and $Q_{\text{max}}$ (mg g$^{-1}$) is the maximum mass of drug removed per gram of complex.

Data fitted well the Langmuir equation for ATO, RST and SIM giving $R^2 = 0.9873$, 0.9748 and 0.9568 for activated charcoal and 0.9223, 0.9408 and 0.9154 for the micelle-clay, respectively (Table 3). The values of $K$ and $Q_{\text{max}}$ parameters for the adsorption isotherm obtained using micelle-clay complex were larger than those corresponding to activated charcoal, suggesting the former as the better adsorbent for ATO, RST and SIM removal.

3.6 Filtration results

ATO, RST and SIM solutions were passed separately through filtering columns, which included the micelle-clay complex or activated charcoal mixed with excess sand at 1:50 ratios (w/w). The results (Table 4) indicate a significant advantage of the micelle-clay filter in removing ATO, RST and SIM compared to the removal by activated charcoal. The removal efficiency of filters filled with activated charcoal and sand was acceptable only for the lowest ATO, RST and SIM concentrations, whereas the micelle clay system removed the drugs completely at the higher concentrations. This finding is in line with the adsorption isotherms, which showed that the micelle-clay complex was more efficient than activated charcoal in adsorbing ATO, RST and SIM from water.
Previously reported experiments demonstrated the poor capability of activated carbon filters towards removing anionic and certain neutral pollutants.[30, 30, 34-36, 47]

Khamis et al. (2012) [30] concluded that the incorporation of micelle-clay filters in sewage treatment systems with loose tertiary capability can be a promising technology. In a recent paper, Karaman et al. (2012) [31] showed that micelle-clay filters were more efficient towards removal of diclofenac from wastewater than activated carbon.

Polubesova et al. (2005) [34] found very efficient removal of three anionic herbicides (imazaquin, sulfentrazone, sulfosulfuron) and 4 neutral pollutants (alachlor, acetochlor, chlorotoluron and bromacil) by micelle–clay complexes in aqueous dispersions. In another study [35] column filters filled with a mixture of quartz sand and micelle–clay complex provided very efficient result for the removal of the antibiotics tetracycline and sulfonamide from water.

Moreover, Zadaka et al. (2007) [36] tested column filters with either a mixture of quartz sand and organic micelle – montmorillonite or zeolite; both filters were capable to remove well anionic pollutants such as sulfosulfuron, imazaquin and sulfentrazone, and neutral compounds such as bromacil and chlorotoluron from aqueous environments; in contrast a filter filled with the same weight of activated carbon and sand removed only partially these pollutants.

More recently, Khalaf et al. (2013) [47] suggested that the integration of clay-micelle complex filters in existing WWTPs may be helpful for improving removal efficiency of recalcitrant residues of non- steroid anti-inflammatory drugs (NSAIDs).

It can be argued that in addition to ATO, RST and SIM residues wastewater usually includes other recalcitrant organic pollutants. In such cases GAC filters can be used as first stage tertiary process to remove the majority of neutral pollutants, and additional micelle-clay filters can be adopted as second stage to eliminate anionic pollutants, and neutral compounds not retained by GAC filters.

4. Conclusion

The stability studies of Atorvastatin, Rosuvastatin and Simvastatin revealed that the three statins were unstable both in water and sludge environments. In addition, it was found that the rate of degradation in sludge was higher than in water due to the presence of many bacterial
species having a variety of enzymes which can catalyze the degradation processes for the mentioned three statins. It should be worth noting that the higher degradation rate found for Simvastatin compared to that of Atorvastatin and Rosuvastatin can be attributed to the relatively unstable lactone ring present in Simvastatin.

The filtration study involving an advanced wastewater treatment plant utilizing ultrafiltration, activated carbon and reverse osmosis demonstrated that activated carbon and RO are efficient in removing the commonly used anti-inflammatory Atorvastatin, Rosuvastatin and Simvastatin from wastewater. But fouling problems due to the high bacterial load in the sludge and the production of brine cannot be avoided. For this reason a filter based on micelle-clay complex, (ODTMA)-montmorillonite, was tested and found to be very efficient in removing all three statins from solution in the mgL⁻¹ or µgL⁻¹ ranges. The large effectiveness and removal capacity are due to a relatively high affinity of adsorption of the anionic Atorvastatin, Rosuvastatin and Simvastatin by the large number of positively charged and hydrophobic sites of the micelle-clay complex based on ODTMA. Furthermore, pilot experiments are needed to evaluate if filters based on micelle-clay could be included in WWTPs aiming at the reduction of membrane’s use or at enhancing their efficiency and prolonging their life.

Acknowledgments
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RK and MK acknowledge the generous grant for supporting part of this work in the framework of the program MENA, project ‘Upgrading Treatment Processes to Improve Effluent Quality for Irrigation’- Prime Contract/TO No.: AID-OAA-T0-11-00049.
This work was partially supported by a generous grant from Sanofi Pharmaceutical Company (France) managed through Peres Center for Peace.

References
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[11] Heberer T. Tracking persistent pharmaceutical residues from municipal sewage to drinking...


Table 1: Removal of ATO, RST and SIM from wastewater by different treatment units in Al-Quds WWTP; average values of three replicates.

<table>
<thead>
<tr>
<th>Sample description</th>
<th>Sampling site</th>
<th>Concentration of ATO, RST &amp; SIM mg L⁻¹</th>
<th>Removal %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>as in Figure S1</td>
<td>Means ± S.D.</td>
<td>Means ± S.D.</td>
</tr>
<tr>
<td>The initial concentration of drug in storage tank (after addition of drug)</td>
<td>1</td>
<td>1.0 ± 0.17</td>
<td>0.92 ± 0.04</td>
</tr>
<tr>
<td>UF-HF influent</td>
<td>2</td>
<td>0.85 ± 0.02</td>
<td>0.78 ± 0.03</td>
</tr>
<tr>
<td>UF-HF brine produced</td>
<td>3</td>
<td>0.51 ± 0.03</td>
<td>0.397 ± 0.02</td>
</tr>
</tbody>
</table>

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Table 2: Degradation rates and half-lives of ATO, RST and SIM in sludge and pure water and determination coefficients (R²).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Medium</th>
<th>k (s⁻¹)</th>
<th>Half-life (d)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATO</td>
<td>sludge</td>
<td>2.2×10⁻⁷</td>
<td>36.3</td>
<td>0.9538</td>
</tr>
<tr>
<td></td>
<td>water</td>
<td>1.9×10⁻⁸</td>
<td>433.1</td>
<td>0.9557</td>
</tr>
<tr>
<td>RST</td>
<td>sludge</td>
<td>1.8×10⁻⁷</td>
<td>45.9</td>
<td>0.9962</td>
</tr>
<tr>
<td></td>
<td>water</td>
<td>2.2×10⁻⁸</td>
<td>364.1</td>
<td>0.9177</td>
</tr>
<tr>
<td>SIM</td>
<td>sludge</td>
<td>1.8×10⁻⁶</td>
<td>4.4</td>
<td>0.9483</td>
</tr>
<tr>
<td></td>
<td>water</td>
<td>6.2×10⁻⁷</td>
<td>12.9</td>
<td>0.9892</td>
</tr>
</tbody>
</table>

Table 3: Langmuir adsorption parameters (K and Q_max) and determination coefficients (R²) obtained from the adsorption of ATO, RST and SIM on the micelle-clay complex and activated charcoal.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Adsorbent</th>
<th>K (L mg⁻¹)</th>
<th>Q_max (mg g⁻¹)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATO</td>
<td>Micelle-clay complex</td>
<td>10.7</td>
<td>23.2</td>
<td>0.9223</td>
</tr>
<tr>
<td></td>
<td>Activated charcoal</td>
<td>5.0</td>
<td>9.1</td>
<td>0.9873</td>
</tr>
<tr>
<td>RST</td>
<td>Micelle-clay complex</td>
<td>8.2</td>
<td>29.4</td>
<td>0.9408</td>
</tr>
<tr>
<td></td>
<td>Activated charcoal</td>
<td>7.4</td>
<td>27.3</td>
<td>0.9748</td>
</tr>
<tr>
<td>SIM</td>
<td>Micelle-clay complex</td>
<td>8.4</td>
<td>24.4</td>
<td>0.9154</td>
</tr>
<tr>
<td></td>
<td>Activated charcoal</td>
<td>6.5</td>
<td>11.9</td>
<td>0.9568</td>
</tr>
</tbody>
</table>
Table 4: Removal of ATO, RST and SIM by filtration of 1L of pure water solutions (100, 10, 1.0, 0.01 mg L⁻¹) through laboratory filtering columns, which included either MC or GAC mixed with excess sand at 1:50 (w/w) ratio; means of three replicates.

<table>
<thead>
<tr>
<th>Initial concentration (mg L⁻¹)</th>
<th>Column type a</th>
<th>Average eluted concentration ± S.D: (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ATO</td>
</tr>
<tr>
<td>100</td>
<td>MC</td>
<td>b.l.d.</td>
</tr>
<tr>
<td>100</td>
<td>GAC</td>
<td>62.2±2.5</td>
</tr>
<tr>
<td>10</td>
<td>MC</td>
<td>b.l.d.</td>
</tr>
<tr>
<td>10</td>
<td>GAC</td>
<td>2.8±0.48</td>
</tr>
<tr>
<td>1.0</td>
<td>MC</td>
<td>b.l.d.</td>
</tr>
<tr>
<td>1.0</td>
<td>GAC</td>
<td>0.25±0.05</td>
</tr>
<tr>
<td>0.01</td>
<td>MC</td>
<td>b.l.d.</td>
</tr>
<tr>
<td>0.01</td>
<td>GAC</td>
<td>b.l.d.</td>
</tr>
</tbody>
</table>

*aFlow rate, 2 mL min⁻¹; temperature, 25 °C; pH, 7.2
b.l.d., below the detection limit of the analytical method used.
List of Figures

Fig. 1. Chemical structures of Atorvastatin (1), Rosuvastatin (2), Simvastatin (3) and Rosuvastatin lactone (RSTL) (4), Octadecyltrimethylammonium ion (5).

Fig. 2. Kinetics of ATO (A) and SIM (B) degradation in pure water (plot a) (♦) and activated sludge (plot b) (■). Data are reported as natural logarithm of concentrations (C(t)) vs. time. Initial concentration (C(0)) = 100 mg L⁻¹. Plotted values are the means of three replicates; bars represent the standard deviations calculated for each average value, T = 25 °C.

Fig. 3. Atorvastatin metabolism to ortho or para hydroxy atorvastatin by CYP3A4.

Fig. 4 -Extracted ion chromatograms (XICs) by LC/ESI-FTICR MS acquired in negative ion mode of ATO solution (a), RST solution (b) and in negative and positive ion mode of SIM (c) after one month of biodegradation. The ions monitored are displayed in each trace and correspond to the most abundant protonated or deprotonated molecules, [M+H]^+ or [M-H]⁻, using a restricted window of ±0.0010 m/z unit centered on each selected ion.

Fig. 5. Chemical structures of Atorvastatin biodegradation products. Ortho, para-dihydroxyatorvastatin, (7-[2-(4-Fluoro-phenyl)-4-(4-hydroxy-phenylcarbamoyl)-5-isopropyl-3-phenyl-pyrrol-1-yl]-3,5-dihydroxy-heptanoic acid), C₃₃H₃₅FN₂O₇ with exact m/z 589.23555 (6); para-hydroxy Atorvastatin, (7-[2-(4-Fluoro-phenyl)-4-(4-hydroxy-phenylcarbamoyl)-5-isopropyl-3-phenyl-pyrrol-1-yl]-3,5-dihydroxy-heptanoic acid), C₃₃H₃₅FN₂O₆ with exact m/z 573.24064 (7); 7-[4-(2,4-Dihydroxy-phenylcarbamoyl)-2-(4-fluoro-phenyl)-3-phenyl-pyrrol-1-yl]-3,5-dihydroxy-heptanoic acid, C₃₀H₂₇FN₂O₇ with exact m/z 547.18860 (8); 7-[4-(2,4-Dihydroxy-phenylcarbamoyl)-2-(4-fluoro-phenyl)-3-phenyl-pyrrol-1-yl]-3,5-dihydroxy-heptanoic acid, C₃₀H₂₇FN₂O₇ with exact m/z 545.17295 (9); 5-(4-Fluoro-phenyl)-1-(3-hydroxy-5-oxo-pentyl)-2-isopropyl-4-phenyl-1H-pyrrole-3-carboxylic acid (4-hydroxy-phenyl)-amide, C₃₁H₃₁FN₂O₄ with exact m/z 513.21951 (10) and Simvastatin acid C₂₅H₄₀O₆ with exact m/z 435.27560 (11).
Fig. 1.
Fig. 2.

Fig. 3.
Fig. 4.
Fig. 5.