Therapeutic Levels of Levonorgestrel Detected in Blood Plasma of Fish: Results from Screening Rainbow Trout Exposed to Treated Sewage Effluents

JERKER FICK,*,† RICHARD H. LINDBERG,† JARI PARKKONEN,‡ BJÖRN ARVIDSSON,§ MATS TYSKLIND,† AND D. G. JOAKIM LARSSON||

Department of Chemistry, Umeå University, SE-901 87 Umeå, Sweden, Department of Zoology, University of Gothenburg, Box 463, SE-405 30 Gothenburg, Sweden, CRBN Defence and Security, Swedish Defence Research Agency, SE-901 82 Umeå, Sweden, and Department of Neuroscience and Physiology, the Sahlgrenska Academy at the University of Gothenburg, SE-405 30 Gothenburg, Sweden

Received November 13, 2009. Revised manuscript received February 24, 2010. Accepted March 1, 2010.

Pharmaceuticals are found in surface waters worldwide, raising concerns about effects on aquatic organisms. Analyses of pharmaceuticals in blood plasma of fish could provide means to assess risk for pharmacological effects, as these concentrations could be compared with available human therapeutic plasma levels. In this study we investigated if fish exposed to sewage effluents have plasma concentrations of pharmaceuticals that are approaching human therapeutic levels. We also evaluated how well the bioconcentration of pharmaceuticals into fish blood plasma can be predicted based on lipophilicity. Rainbow trout were exposed to undiluted, treated sewage effluents at three sites in Sweden for 14 days. Levels of 25 pharmaceuticals in blood plasma and effluents were analyzed with liquid chromatography-mass spectrometry/mass spectrometry and gas chromatography-high resolution mass spectrometry. The progestin pharmaceutical levonorgestrel was detected in fish blood plasma at concentrations (8.5−12 ng mL^{-1}), exceeding the human therapeutic plasma level. In total 16 pharmaceuticals were detected in fish plasma at concentrations higher than 1/1000 of the human therapeutic plasma concentration. Twenty-one pharmaceuticals were detected in either plasma or effluent, and 14 were detected in both compartments, allowing plasma bioconcentration factors to be determined. For 11 of these, theoretically calculated and experimentally measured values were in reasonably good agreement. However a few drugs, including levonorgestrel, did not bioconcentrate according to the screening model used. This study shows that rainbow trout exposed to sewage effluents have blood plasma levels of pharmaceuticals similar to human therapeutic concentrations, suggesting a risk for pharmacological effects in the fish. There is a particular concern about effects of progestin pharmaceuticals. For levonorgestrel, the measured effluent level (1 ng/L) was higher than water levels shown to reduce the fertility of fish.

Introduction

Residues of human and veterinary pharmaceuticals have been detected in surface waters worldwide (*1*−*6*) and in some cases also in tissues of aquatic organisms (*7*−*10*). These findings have raised concerns about their potential for environmental effects (*11*−*13*). Even though pharmaceuticals are most often detected at trace concentrations in the aquatic environment, typically 0.001−1 μg L^{-1}, their biological potency makes their presence an issue of concern. Due to the conservative nature of physiological processes, many aquatic species possess similar drug target molecules to those found in humans (*14*), suggesting a potential for pharmacological interactions of human drugs in exposed nontarget species.

Huggett et al. presented a screening-level approach to predict the likelihood for pharmacological interactions in aquatic species at a given water concentration of an active pharmaceutical ingredient (*15*). This approach, often referred to as "the fish plasma model", assumes a functionally conserved molecular drug target in the wildlife species of interest and hence that the target will be affected at roughly the same blood plasma concentration of the pharmaceutical as in humans. Studying fish is motivated as their physiology is often remarkably similar to that of humans, particularly on the molecular level, including a strong conservation of most drug targets (*14, 16*). In cases where the target protein is not present in the fish, or not sufficiently similar to the mammalian counterparts to allow the drug to bind, the fish will likely be less sensitive, and this basic approach may overestimate the risk.

The model proposed by Huggett et al. compares a theoretically estimated fish plasma concentration with a known human/mammalian therapeutic plasma concentration (*15*). This equation yields "effect ratios", i.e. the ratio between the plasma concentrations of humans treated with a therapeutic dose of the pharmaceutical compared to the estimated plasma concentration in fish under a given water concentration, i.e. effect ratio = HTPC/FssPC, where the HTPC is the human therapeutic plasma concentration and FssPC is the fish steady state plasma concentration. Strictly speaking, the term effect ratio is somewhat misleading as it refers to a ratio of two concentrations, thus we will use the term "concentration ratio" from here on. If the concentration ratio is lower than 1 then the concentration in the exposed fish is higher or equal to the concentration known to give a pharmacological response in humans, thus there is an obvious risk that the fish could be affected as well. It has to be emphasized that this model only reflects the probability of a pharmacological effect and not whether this effect is adverse or not.

In the equation used to predict the bioconcentration factor from water to fish blood (plasma BCF), the only information used is a measure of the lipophilicity ($\log K_{ow}$) of the pharmaceutical. For nonpolar contaminants this gives a rather good estimate (*17*); however, it is not known how good a predictor the $\log K_{ow}$ of the BCF for pharmaceuticals is in general. Ionization plays a vital role in the process of bioconcentration, and several exposure studies of weak acids and bases have shown a pH dependent uptake rate and a

* Corresponding author phone: 46-90-7869324; fax: 46-90-7867655; e-mail: jerker.fick@chem.umu.se.
† Umeå University.
‡ Department of Zoology, University of Gothenburg.
§ Swedish Defence Research Agency.
|| The Sahlgrenska Academy at the University of Gothenburg.

10.1021/es903440m

greater bioavailability of the neutral form (*18, 19*). Surprisingly high uptake rates have been observed for some ionized chemicals however (*20*), and methods to estimate the bioconcentration of ionized compounds have been presented accordingly (*21, 22*). In this study we compared empirical data on plasma BCF with predictions based on the screening-level equation as presented in ref *15*. Brown et al. also tested this equation for a small set of pharmaceuticals and showed that measured plasma BCFs in general were similar or lower than those modeled and that the plasma BCFs varied between the investigated effluents for some pharmaceuticals (*9*).

Our aim was to investigate if fish exposed to sewage effluents have plasma concentrations of pharmaceuticals that are approaching human therapeutic levels, as this would indicate a significant risk for pharmacological effects. To achieve this we measured the levels of 25 pharmaceuticals in plasma of fish exposed to the final effluent from three major sewage treatment plants (STPs) in Sweden. Another aim was to investigate how well the plasma BCF could be predicted using the model proposed by Hugget et al. (*15*).

Materials and Methods

Field Exposures. The three STPs (Stockholm, Henrikdal STP; Gothenburg, Gryab STP; Umeå STP) chosen for field exposures encompass a range of size, treatment technologies, and geographic locations, thus making the results more widely applicable. The effluent treatment includes chemical removal of phosphorus, primary clarification, active sludge treatment with nitrogen removal (except Umeå), and secondary clarification. As for sludge, the final products from all STPs are anaerobic digested sludge. For more information regarding each STP see Table S1, Supporting Information.

Juvenile rainbow trout (*Oncorhynchus mykiss*) of both sexes (average weight approximately 100 g) were obtained from Antens fiskodling AB, Sweden (for exposures at Henriksdal and Gryaab) and Umlax AB, Sweden (for Umeå). Fish were exposed to aerated, undiluted, treated effluent in tanks with a flow-through system in Gothenburg, Stockholm, and Umeå between February 24 and March 7, March 3 and March 16, and March 30 and May 13, 2008, respectively. The average water temperatures during the exposures were 9.9, 13.5, and 9.5 °C, respectively and pH was stable at 7.5−8.0. Samples of treated effluent were taken daily using flow proportional sampling at all STPs. During the exposures, the fish were not fed. At the end of the exposure the fish were stunned by a blow to the head and blood (0.1−1.0 mL fish^{-1}) taken from the caudal vein using a heparinized ice-cooled syringe. After centrifuging (2 min at 6000 rpm), the plasma was snap-frozen in liquid nitrogen and then transferred to a −18 °C freezer prior to analysis. Experiments were carried out according to animal ethics permit no. 36-2007 to D. G. J. Larsson.

Calculation of Plasma Bioconcentration Factors and Plasma Concentration Ratios. Theoretical plasma BCFs ($P_{\text{blood:water}}$) were estimated for each pharmaceutical by the equation; $\log P_{\text{blood:water}} = 0.73 \times \log K_{\text{ow}} - 0.88$, using the $\log K_{\text{ow}}$ of the uncharged form (*16*). Estimated log *P* values ($\log K_{\text{ow}}$), shown in Table S2, Supporting Information, were retrieved from EPI Suite KowWin program (http://www.epa.gov/oppt/exposure/pubs/episuitedl.htm). Actual plasma BCFs were based on measured fish plasma levels at the end of the exposure and the average measured water concentration of the pharmaceuticals during the 14-day exposure period. In calculating plasma concentration ratios, the lowest found literature human *C*max values (maximum blood plasma concentration after a normal dose) were used as human therapeutic plasma concentration (Table S2, Supporting Information) thus resulting in more conservative estimates. Maximum *C*max values found were at most five times higher than the ones used, thus the choice of Cmax value may have some but limited impact on the estimation of the plasma concentration ratios.

Chemicals. Bezafibrate, carbamazepine, clomipramine, diclofenac, diltiazem, fexofenadine, fluoxetine, haloperidol, ibuprofen, ketoconazole, ketoprofen, memantine, mianserin, naproxen, oxazepam, risperidone, sertraline, telmisartan, and verapamil were obtained from Sigma-Aldrich (Steinheim, Germany) and were all classified as HPLC grade (>98%). Cilazapril, levonorgestrel, meclozine, megestrol, orphenadrine, and tramadol were bought from European Pharmacopoeia (Strasbourg Cedex 1, France); all were classified as HPLC grade (>98%). *N,O*-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + TMCS 99:1, Sylon BFT) and *N*-trimethylsilylimidazole (TMSI) of derivatization grade were obtained from Sigma-Aldrich (Steinheim, Germany). Methanol (HPLC-grade) was purchased from JT Baker (Deventer, The Netherlands), and sulfuric acid was purchased from Merck (Darmstadt, Germany). The purified water (resistivity, 18.2 MΩ cm) was prepared by an ELGA MAXIMA HPLC ultra pure water system (ELGA, High Wycombe Bucks, England), equipped with a UV radiation source. D10-carbamezepine, D5-fluoxetine, ^{13}C3D3- naproxen, and ^{13}C3-ibuprofen were obtained from Cambridge Isotope Laboratories (Andover, MA, U.S.A.), while D5-oxazepam was obtained from Sigma-Aldrich.

Pretreatment of Plasma and Sewage Effluent Samples. Two separate pools of plasma, each based on six individual fish, were analyzed from each site. The diluted plasma (0.5 mL with 1.5 mL 1% aqueous formic acid) was filtered through a 0.45 μm membrane filter (MF, Millipore, Sundbyberg, Sweden). Sewage effluent (100 mL) was also filtered through a 0.45 μm membrane filter (MF, Millipore, Sundbyberg, Sweden) and then acidified to pH 3 using sulfuric acid. Five hundred nanograms of each surrogate standard was added to each plasma and effluent sample. Solid phase extraction (SPE) columns (Oasis HLB, 200 mg, Waters Corporation, Milford, MA, U.S.A.) were preconditioned and equilibrated with 5.0 mL of methanol and 5.0 mL of deionized water. Both plasma and effluent samples were applied to the SPE columns at a flow rate of 5 mL min^{-1}; water with 5% methanol was used to wash the SPE column before eluting with 5 mL of methanol. Eluate was collected in 10 mL vials, evaporated to 20 μL under a gentle air stream, and dissolved in 5% acetonitrile in water to a final volume of 1.0 mL.

Liquid Chromatography. Bezafibrate, carbamazepine, cilazapril, clomipramine, diltiazem, fexofenadine, fluoxetine, haloperidol, ketoconazole, levonorgestrel, meclozine, megestrol, memantine, mianserin, orphenadrine, oxazepam, risperidone, sertraline, telmisartan, and verapamil were analyzed using liquid chromatography mass spectrometry, with D10-carbamezepine, D5-fluoxetine, and D5-oxazepam as surrogate standards. A microdialysis autosampler (CMA Microdialysis AB, Stockholm, Sweden) was used for sample introduction using a 40 μL loop. An injection needle and a loop were cleaned with a wash solution (50% (v/v) methanol in water) between runs. For the analytical separation a LC Packings PepMap100 column, 150 × 0.300 mm i.d., 3 mm particle size 100 Å pore size (Dionex/LC Packings, Amsterdam, Netherlands), was used. A 1 mL min^{-1} flow was delivered by a Waters Alliance LC pump (Waters, Milford, U.S.A.) and was split (1:200) prior to the autosampler. Water and acetonitrile (both with 0.1% formic acid) were used as the mobile phase, and the sample loading (40 μL) onto the analytical column was performed by an isocratic flow of 95% water and 5% acetonitrile, at a flow rate of 5 μL min^{-1}. After loading the following LC gradient program was performed: constant flow of 5 μL min^{-1}; initial isocratic flow of 95% water and 5% acetonitrile held for 2 min; 3% min^{-1} rise of acetonitrile to 80% and hold 3 min. Before each run the column was equilibrated at 95% water and 5% acetonitrile for 7 min.

Analytes were detected using a Waters/Micromass Quattro Triple quadropole mass spectrometer (Waters/Micromass, Milford, U.S.A.) in selective reaction monitoring (SRM) mode with one channel for each analyte. Eluted species were ionized by positive electrospray ionization (ESI) at an ion source voltage of 3.1 kV. Each SRM channel had a 100 ms dwell time and individual cone voltages and collision energies that were optimized manually. Samples were quantified using 5 point calibration graphs. The liquid chromatography mass spectrometry parameters and limit of quantification (LOQ) in fish plasma and sewage water are presented in Table S3, Supporting Information.

Gas Chromatography. Diclofenac, ibuprofen, ketoprofen, naproxen, and tramadol were analyzed using gas chromatography mass spectrometry, with ^{13}C3D3-naproxen and ^{13}C3-ibuprofen as surrogate standards. Samples were evaporated until completely dry and reconstituted in 50 μL of pyridine and transferred to 250 μL glass inserts. Twenty-five microliters of BSTFA with 1% TMCS and 1% TMSI was added for derivatization at 60 °C for 45 min (*23*). The TMS derivatives were analyzed on a VG AutoSpec high resolution mass spectrometer (Fisons Instruments, Manchester, UK). The injector was operated in splitless mode at 275 °C. The column used was a J&W Scientific DB-5 M (30 m × 0.25 mm i.d. × 0.25 μm film thickness) capillary column with helium as carrier gas at a constant flow of 1.2 mL min^{-1}. The GC temperature program was as follows: constant flow of 40 cm/s He; initial temperature 90 °C held for 3 min; 25 °C min^{-1} rise to 180 °C; 5 °C min^{-1} to 300 °C and hold 5 min. The ion source and transfer line temperatures were 250 °C. All mass data were obtained using electron ionization at 30 eV and single ion monitoring mode. Identification was conducted by comparison of retention times and the ratio between quantification and qualification ions, relative to those of derivatized authentic standards. Samples were quantified using 5 point calibration graphs. The gas chromatography mass spectrometry parameters and limit of quantification (LOQ) in fish plasma and sewage water are presented in Table S4, Supporting Information.

Quality Assurance/Quality Control. Samples were analyzed in duplicate. Plasma from nonexposed rainbow trout was used as blank samples. Possible memory effects were evaluated by a blank injection of either Milli-Q water or blank fish plasma, after standard samples of varying concentrations. Additional injections of "blank" plasma from unexposed fish were used to evaluate the system selectivity, where an absence of the detection signal indicates that no other species in the matrix interfere with the detection. Further validation of the liquid chromatography system setup was done to evaluate possible cross-talk effects due to chosen interchannel dwell times. This evaluation was done by studying "dummy" channels that consisted of either a false mother ion and a true daughter ion channel or the opposite. Responses at the same retention time in the "dummy"-channels would indicate cross-talk. All analyses using gas chromatography were made at high resolution to increase selectivity. Standards were analyzed, using both liquid and gas chromatography, in a wide concentration range (0.05 ng mL^{-1} to 500 ng mL^{-1}) and were used for evaluating the linearity, sensitivity quantification limit (LOQ) defined as 10 times the standard deviation of the blank, reproducibility of retention, precision as repeatability, and column stability. Method recoveries were monitored by spiking the standard solution to fish plasma and sewage effluent samples. Analyte addition was made with the criteria that the spiking would be at a level at least three times the original concentration.

Results and Discussion

The use of SRM (LC) and high resolution mass spectrometry (GC) showed high selectivity, and in most cases almost no background signal could be detected. In addition, no memory effects or cross-talk could be detected. Both the gas and liquid chromatography were stable throughout the analysis, and all retention times were within 2% of the standards. Absolute recoveries in fish plasma and sewage effluent are shown in Table S5, Supporting Information. Recoveries ranged from 26% to 154% with an average of 99% in fish plasma and 77% in sewage effluent and an average relative standard deviation of 13% and 11%, respectively.

TABLE 1. Concentrations of Selected Pharmaceuticals in Fish Plasma (Mean *N* = 2)

	Umeå (ng mL^{-1})	Stockholm (ng mL^{-1})	Gothenburg (ng mL^{-1})
bezafibrate	<LOQ	<LOQ	<LOQ
carbamazepine	0.9	0.3	1.0
cilazapril	<LOQ	0.1	0.7
diclofenac	20	2.2	7.4
diltiazem	<LOQ	<LOQ	0.9
fexofenadine	<LOQ	<LOQ	<LOQ
haloperidol	<LOQ	<LOQ	1.2
ibuprofen	13	5.5	102
ketoprofen	107	37	15
levonorgestrel	12	8.5	<LOQ
meclozine	<LOQ	0.1	0.7
memantine	<LOQ	<LOQ	2.3
mianserin	<LOQ	<LOQ	<LOQ
naproxen	36	33	46
orphenadrine	<LOQ	<LOQ	0.9
oxazepam	0.4	0.2	0.7
risperidone	0.4	0.3	2.4
sertraline	1.2	1.1	<LOQ
telmisartan	<LOQ	<LOQ	<LOQ
tramadol	1.2	1.1	1.9
verapamil	<LOQ	<LOQ	0.7

Analytical concentrations of pharmaceuticals detected in fish blood plasma are given in Table 1. Fish plasma levels were within 1/1000 of the corresponding human therapeutic plasma concentration for 16 drugs. For levonorgestrel, fish plasma levels exceeded human therapeutic concentrations by a factor of 4 (Figure 1). The fish plasma levels of haloperidol, risperidone, and cilazapril were all higher than a tenth of the human therapeutic plasma concentrations (Figure 1).

In this screening study we found fish that had blood plasma levels of levonorgestrel, a synthetic steroid with a progesterone-like activity, exceeding human therapeutic levels. Progesterone receptors are conserved between fish and humans (*14*), also on a functional level (*24*−*26*), suggesting that the concentrations found in the fish are sufficiently high for a receptor interaction. Normally, fish are not exposed to 100% effluent as some dilution occurs in the recipients; however, there are many streams and rivers that are heavily effluent-dominated (*27*). Given that the plasma levels found were up to 4 times higher than human therapeutic levels, some dilution of effluent could likely occur without fish plasma levels dropping below human therapeutic levels. It should also be stressed that there are many different pharmaceuticals that act via the same receptors as levonorgestrel. For most of these pharmaceuticals, environmental concentrations have not been investigated nor their potential to bioconcentrate into fish. However, if present, they are expected to act in an additive manner as shown for e.g. estrogenic chemicals (*28*). All investigated STPs use activated sludge treatment, thus we do not expect that they represent the low end of treatment efficiency. Taken together, it is plausible that progestin pharmaceuticals cause pharmacological effects in fish living downstream from some sewage treatment plants.

Four of the 17 pharmaceuticals that were detected in fish plasma had a plasma concentration ratio below 10, and more

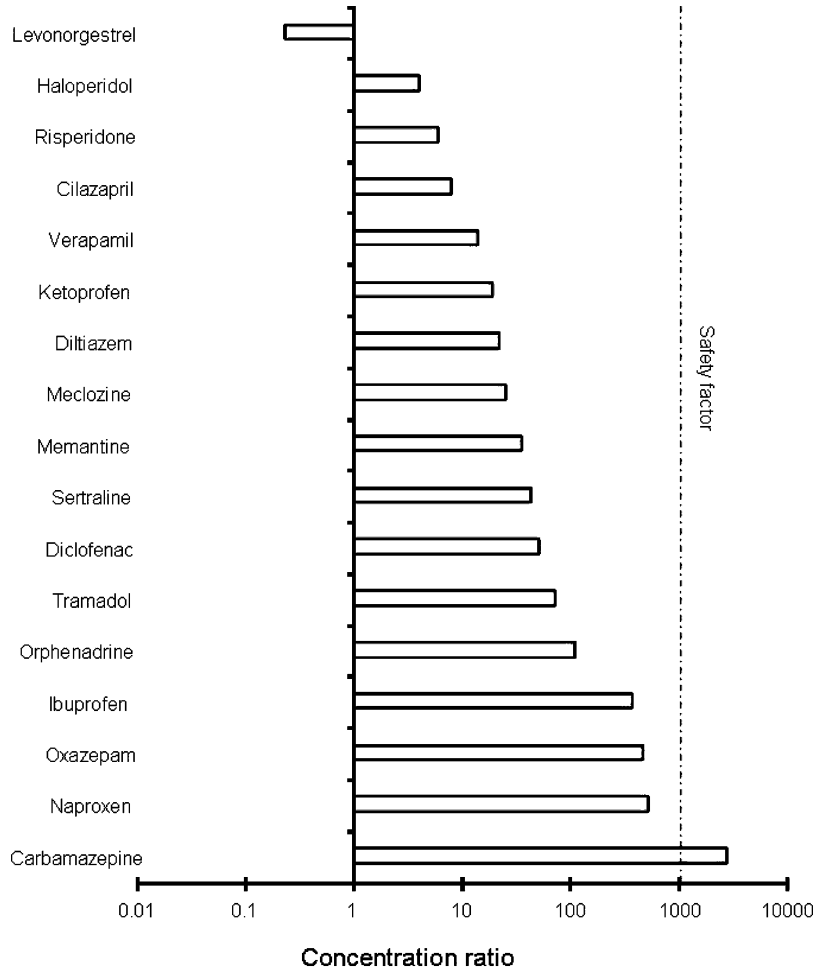

FIGURE 1. Average concentration ratios for the pharmaceuticals detected in fish plasma. Calculated from data in Table 2 and Table S2. The indicated safety factor of 1000 was suggested by Huggett et al. (*15*).

TABLE 2. Concentrations of Pharmaceuticals in the Sewage Treatment Plants, Two Week Average

	Umeå (ng L^{-1})	Stockholm (ng L^{-1})	Gothenburg (ng L^{-1})
bezafibrate	3	9	8
carbamazepine	326	388	236
cilazapril	<LOQ	<LOQ	<LOQ
diclofenac	701	881	720
diltiazem	36	36	37
fexofenadine	38	146	101
haloperidol	<LOQ	<LOQ	374
ibuprofen	625	95	2169
ketoprofen	2220	1319	4268
levonorgestrel	1	<LOQ	<LOQ
meclozine	<LOQ	<LOQ	<LOQ
memantine	10	13	14
mianserin	10	<LOQ	<LOQ
naproxen	1615	1159	1792
orphenadrine	5	8	14
oxazepam	110	277	202
risperidone	<LOQ	<LOQ	<LOQ
sertraline	<LOQ	8	<LOQ
telmisartan	<LOQ	191	104
tramadol	359	479	738
verapamil	1	3	4

than half had a ratio below 100 (Figure 1). Huggett et al. (*15*) suggested a safety factor of 1000 for initial assessments due to, for example, variable plasma BCFs and sensitivities between species. Sixteen of the 17 pharmaceuticals detected in fish plasma had a plasma concentration ratio below 1000, with carbamazepine as the only exception (Figure 1). It should be stressed once more that the model used in this study only estimates the probability of a pharmacological effect and if these effects are adverse or not has to be studied further. Also, most of the investigated drugs are used in high volumes and/or have a moderately high lipophilicity, thus the probability to reach a certain plasma concentration ratio should not simply be extrapolated to all other drugs on the market. Nevertheless, the results indicate that some pharmaceuticals will bioconcentrate in aquatic organisms exposed to sewage effluent to levels relatively close to human therapeutic levels.

Analytical concentrations of pharmaceuticals detected in sewage effluents are given in Table 2. Four pharmaceuticals, clomepramine, fluoxetin, ketoconazole, and megesterol, were not detected in sewage effluent or in fish plasma. Of the 21 pharmaceuticals that were detected, 18 were found in the effluent, 17 in plasma, and 14 were detected in both effluent and plasma, allowing a comparison between predicted and measured plasma BCFs (Table 3).

For 11 out of 14 the pharmaceuticals detected in both water and plasma, theoretically calculated and experimentally measured plasma BCFs were in reasonably good agreement (within an order of magnitude) (Table 3). This could be interpreted as the fish are close to a steady state. This suggests that, for many pharmaceuticals, fairly accurate predictions of their plasma BCFs can be made solely from the $\log K_{ow}$ using the equation by Fitzsimmons et al. (*17*). However, out of the 21 pharmaceuticals detected in either water, plasma,

TABLE 3. Measured and Predicted Plasma Bioconcentration Factors

	Umeå	Stockholm	Gothenburg	predicted
bezafibrate	<17[b]	<5.6[b]	<6.3[b]	168
carbamazepine	2.8	0.8	4.2	6
cilazapril		>100[a]	>700[a]	6
diclofenac	29	2.5	10	93
diltiazem	<139[b]	<139[b]	24	14
fexofenadine	<13[b]	<3.4[b]	<5.0[b]	15
haloperidol			3.2	153
ibuprofen	21	58	47	77
ketoprofen	48	28	3.5	20
levonorgestrel	12000	>8500[a]		46
meclozine		>200[a]	>1400[a]	2521
memantine	<50[b]	<38[b]	164	36
mianserin	<50[b]			37
naproxen	22	28	26	24
orphenadrine	<100[b]	<63[b]	64	61
oxazepam	3.6	0.7	3.5	7
risperidone	>80[a]	>60[a]	>480[a]	47
sertraline	>240[a]	138		959
telmisartan		<5.2	<9.6[b]	178649
tramadol	3.3	2.3	2.6	20
verapamil	<100[b]	<33[b]	175	40

[a] Minimum estimated plasma BCF. Analyte was not detected in the effluent so the concentration was set at the LOQ to estimate minimum plasma BCF. LOQ is shown in Tables S3 and S4 in the Supporting Information. [b] Maximum estimated plasma BCF. Analyte was not detected in fish plasma so the concentration was set at the LOQ to estimate maximum plasma BCF. LOQ is shown in Tables S3 and S4 in the Supporting Information.

or both matrices, four pharmaceuticals had a plasma BCF deviating at least 20-fold from the predicted plasma BCF values. Levonorgestrel and cilazapril bioconcentrated up to 16−260 times more than predicted. Bezafibrate and telmisartan, on the other hand, were not detected in plasma at all despite their presence in effluent, indicating plasma BCFs at least 31-fold and 34000-fold less than predicted for these two drugs, respectively. This shows that other factors than the $\log K_{ow}$ are important for the bioconcentration of some drugs.

Many pharmaceuticals are excreted as conjugates. Some of these are known to deconjugate during sewage treatment, thus bringing back the original pharmaceuticals (*29*). Six of the 18 pharmaceuticals found in fish plasma, i.e. ketoprofen, levonorgestrel, naproxen, memantine, oxazepam, and tramadol, have glucuronide or sulfation conjugates as their major metabolites (*30*). To our best knowledge neither of these conjugates have been detected in treated sewage effluents, and there fate and possible deconjugation in the sewage treatment remain to be studied. However, since these conjugates are by definition much more water-soluble than their unconjugated counterparts, they are expected to bioconcentrate to a much lesser degree. We therefore consider it unlikely that they would contribute significantly to the levels of unconjugated pharmaceuticals found in the fish plasma.

Levonorgestrel. Levonorgestrel is a synthetic progesterone used in contraceptives and in emergency contraceptive pills. The levels found in the fish plasma from Umeå (12 ng mL^{-1}) and Stockholm (8.5 ng mL^{-1}) (Table 1, chromatogram in Figure S1, Supporting Information) should be compared to a human therapeutic plasma concentration of 2.4 ng mL^{-1} (*31*). When used as an emergency contraceptive pill, human plasma levels of levonorgestrel are in the same range as in the fish from Umeå (*32*). Zeilinger et al. (*33*) recently showed that a water concentration of levonorgestrel as low as 0.8 ng L^{-1}, the lowest tested concentration, caused a reduced fertility of exposed adult fathead minnows. The effluent concentration of levonorgestrel in Umeå was 1 ng L^{-1} (Table 2), similar to previous measurements in effluents (*34*), i.e. levels sufficient to impair the reproduction of fish. Levonorgestrel had measured plasma BCF of 12000 which exceeds the predicted value more than 200-fold (Table 3). Miguel-Queralt and Hammond (*35*) recently presented a plausible mechanism for a higher-than expected uptake of sex-steroids in fish, mediated via binding to sex-steroid binding globulins (SSBG) in the gills. Indeed, levonorgestrel was shown to bind with high affinity to the zebrafish SSBG. Experiments with live zebrafish exposed to steroids in water also showed that other ligands to the SSBG (ethinylestradiol and testosterone) were rapidly taken up, whereas cortisol, which has low affinity to the SSBG, remained in the water (*32*). Quite possibly, the SSBG rapidly sequesters sex-steroids entering the gill, preventing them from returning to the surrounding water, thus acting as a trap. This could clearly be an explanation why a concentration of 1 ng/L of levonorgestrel can result in fish plasma levels exceeding human therapeutic levels. The strong bioconcentation found here is in accordance with the concentration−response data reported by Zeilinger et al. (*33*), which could be regarded as indirect indications of a significant uptake of levonorgestrel already at sub ng L^{-1} concentrations. Thus, the two studies corroborate each other. Taken together, they provide strong incentives to further study the environmental fate and potential impact of levonorgestrel and other similarly acting progestins on aquatic vertebrates.

Acknowledgments

The authors thank the employees at the sewage treatment plants for assistance with fish exposure and The Foundation for Strategic Environmental Research and the Swedish Research Council for financial support.

Supporting Information Available

A detailed description of the included sewage treatment plants, gas and liquid chromatography mass spectrometry parameters, and limit of quantifications (LOQs); absolute recoveries, LogP, and human therapeutic plasma concentrations for the included pharmaceuticals; and chromatogram of levonorgestrel in fish plasma. This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Vieno, N. M.; Tuhkanen, T.; Kronberg, L. Seasonal variation in the occurrence of pharmaceuticals in effluents from a sewage treatment plant and in the recipient water. *Environ. Sci. Technol.* **2005**, *39* (21), 8220–8226.

(2) Vieno, N. M.; Tuhkanen, T.; Kronberg, L. Analysis of neutral and basic pharmaceuticals in sewage treatment plants and in recipient rivers using solid phase extraction and liquid chromatography-tandem mass spectrometry detection. *J. Chromatogr., A* **2006**, *1134* (1−2), 101–111.

(3) Nikolaou, A.; Meric, S.; Fatta, D. Occurrence patterns of pharmaceuticals in water and wastewater environments. *Anal. Bioanal. Chem.* **2007**, *387* (4), 1225–1234.

(4) Williams, R. T.; Cook, J. C. Exposure to pharmaceuticals present in the environment. *Drug Inf. J.* **2007**, *41* (2), 133–141.

(5) Khetan, S. K.; Collins, T. J. Human pharmaceuticals in the aquatic environment: A challenge to green chemistry. *Chem. Rev.* **2007**, *107* (6), 2319–2364.

(6) Fick, J.; Söderstrom, H.; Lindberg, R. H.; Phan, C.; Tysklind, M.; Larsson, D. G. J. Contamination of Surface, Ground, and Drinking Water from Pharmaceutical Production. *Environ. Toxicol. Chem.* **2009**, *28* (12), 2522–2527.

(7) Larsson, D. G. J.; Adolfsson-Erici, M.; Parkkonen, J.; Pettersson, M.; Berg, A. H.; Olsson, P. E.; Förlin, L. Ethinyloestradiol - an undesired fish contraceptive? *Aquat. Toxicol.* **1999**, *45* (2−3), 91–97.

(8) Brooks, B. W.; Chambliss, C. K.; Stanley, J. K.; Ramirez, A.; Banks, K. E.; Johnson, R. D.; Lewis, R. J. Determination of select antidepressants in fish from an effluent-dominated stream. *Environ. Toxicol. Chem.* **2005**, *24* (2), 464–469.

(9) Brown, J. N.; Paxeus, N.; Forlin, L.; Larsson, D. G. J. Variations in bioconcentration of human pharmaceuticals from sewage effluents into fish blood plasma. *Environ. Toxicol. Chem.* **2007**, *24* (3), 267–274.
(10) Ramirez, A.; Brain, R.; Usenko, S.; Mottaleb, M.; O'Donnell, J.; Stahl, L.; Wathen, J.; Snyder, B.; Pitt, J. P.-H. P.; Dobbins, L.; Brooks, B.; Chambliss, K. Occurrence of Pharmaceuticals and Personal Care Products in Fish: Results of A National Pilot Study in the United States. *Environ. Toxicol. Chem.* **2009**, *28* (12), 2587–2597.
(11) Fent, K.; Weston, A. A.; Caminada, D. Ecotoxicology of human pharmaceuticals. *Aquat. Toxicol.* **2006**, *76* (2), 122–159.
(12) Crane, M.; Watts, C.; Boucard, T. Chronic aquatic environmental risks from exposure to human pharmaceuticals. *Sci. Total Environ.* **2006**, *367* (1), 23–41.
(13) Poynton, H. C.; Vulpe, C. D. Ecotoxicogenomics: Emerging Technologies for Emerging Contaminants. *J. Am. Water Resour. Assoc.* **2009**, *45* (1), 83–96.
(14) Gunnarsson, L.; Jauhiainen, A.; Kristianssn, E.; Nerman, O.; Larsson, D. G. J. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. *Environ. Sci. Technol.* **2008**, *42* (15), 5807–5813.
(15) Huggett, D. B.; Cook, J. C.; Ericson, J. F.; Williams, R. T. A theoretical model for utilizing mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish. *Hum. Ecol. Risk Assess.* **2003**, *9* (7), 1789–1799.
(16) Owen, S. F.; Giltrow, E.; Huggett, D. B.; Hutchinson, T. H.; Saye, J.; Winter, M. J.; Sumpter, J. P. Comparative physiology, pharmacology and toxicology of beta-blockers: Mammals versus fish. *Aquat. Toxicol.* **2007**, *82* (3), 145–162.
(17) Fitzsimmons, P. N.; Fernandez, J. D.; Hoffman, A. D.; Butterworth, B. C.; Nichols, J. W. Branchial elimination of superhydrophobic organic compounds by rainbow trout (Oncorhynchus mykiss). *Aquat. Toxicol.* **2001**, *55* (1−2), 23–34.
(18) Howe, G. E.; Marking, L. L.; Bills, T. D.; Rach, J. J.; Mayer, F. L. Effects of Water Temperature and Ph on Toxicity of Terbufos, Trichlorfon, 4-Nitrophenol and 2,4-Dinitrophenol to the Amphipod Gammarus-Pseudolimnaeus and Rainbow-Trout (Oncorhynchus-Mykiss). *Environ. Toxicol. Chem.* **1994**, *13* (1), 51–66.
(19) Arnot, J. A.; Gobas, F. A. P. C. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. *Environ. Rev.* **2006**, *14* (4), 257–297.
(20) Erickson, R. J.; Mckim, J. M.; Lien, G. J.; Hoffman, A. D.; Batterman, S. L. Uptake and elimination of ionizable organic chemicals at fish gills: II. Observed and predicted effects of pH, alkalinity, and chemical properties. *Environ. Toxicol. Chem.* **2006**, *25* (6), 1522–1532.
(21) Erickson, R. J.; Mckim, J. M.; Lien, G. J.; Hoffman, A. D.; Batterman, S. L. Uptake and elimination of ionizable organic chemicals at fish gills: I. Model formulation, parameterization, and behavior. *Environ. Toxicol. Chem.* **2006**, *25* (6), 1512–1521.
(22) Fu, W. J.; Franco, A.; Trapp, S. Methods for Estimating the Bioconcentration Factor of Ionizable Organic Chemicals. *Environ. Toxicol. Chem.* **2009**, *28* (7), 1372–1379.
(23) Shareef, A.; Angove, M. J.; Wells, J. D. Optimization of silylation using N-methyl-N-(trimethylsilyl)-trifluoroacetamide, N, O-bis-(trimethyl.silyl)-trifluoroacetamide and N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide for the determination of the estrogens estrone and 17 alpha-ethinylestradiol by gas chromatography-mass spectrometry. *J. Chromatogr., A.* **2006**, *1108* (1), 121–128.
(24) Zhu, Y.; Bond, J.; Thomas, P. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (5), 2237–2242.
(25) Stolte, E. H.; van Kemenade, B. M. L. V.; Savelkoul, H. F. J.; Flik, G. Evolution of glucocorticoid receptors with different glucocorticoid sensitivity. *J. Endocrinol.* **2006**, *190* (1), 17–28.
(26) Bridgham, J. T.; Carroll, S. M.; Thornton, J. W. Evolution of hormone-receptor complexity by molecular exploitation. *Science* **2006**, *312* (5770), 97–101.
(27) Brooks, B. W.; Riley, T. M.; Taylor, R. D. Water quality of effluent-dominated ecosystems: ecotoxicological, hydrological, and management considerations. *Hydrobiologia* **2006**, *556*, 365–379.
(28) Brian, J. V.; Harris, C. A.; Scholze, M.; Backhaus, T.; Booy, P.; Lamoree, M.; Pojana, G.; Jonkers, N.; Runnalls, T.; Bonfa, A.; Marcomini, A.; Sumpter, J. P. Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. *Environ. Health Perspect.* **2005**, *113* (6), 721–728.
(29) Celiz, M. D.; Tso, J.; Aga, D. S. Pharmaceutical Metabolites in the Environment: Analytical Challenges and Ecological Risks. *Environ. Toxicol. Chem.* **2009**, *28* (12), 2473–2484.
(30) *Martindale: The Complete Drug Reference*; electronic version (edition 100125); Pharmaceutical Press: London, 2010.
(31) Endrikat, J.; Blode, H.; Gerlinger, C.; Rosenbaum, P.; Kuhnz, W. A pharmacokinetic study with a low-dose oral contraceptive containing 20 mu g ethinylestradiol plus 100 mu g levonorgestrel. *Eur. J. Contracept. Reprod. Health Care* **2002**, *7* (2), 79–90.
(32) Johansson, E.; Brache, V.; Alvarez, F.; Faundes, A.; Cochon, L.; Ranta, S.; Lovern, M.; Kumar, N. Pharmacokinetic study of different dosing regimens of levonorgestrel for emergency contraception in healthy women. *Hum. Reprod.* **2002**, *17* (6), 1472–1476.
(33) Zeilinger, J.; Steger-Hartmann, T.; Maser, E.; Goller, S.; Vonk, R.; Länge, R. Effects of Synthetic Gestagens on Fish Reproduction. *Environ. Toxicol. Chem.* **2009**, *28* (12), 2663–2670.
(34) Petrovic, M.; Sole, M.; de Alda, M. J. L.; Barcelo, D. Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: Integration of chemical analysis and biological effects on feral carp. *Environ. Toxicol. Chem.* **2002**, *21* (10), 2146–2156.
(35) Miguel-Queralt, S.; Hammond, G. L. Sex hormone-binding globulin in fish gills is a portal for sex steroids breached by xenobiotics. *Endocrinology* **2008**, *149* (9), 4269–4275.

ES903440M

Therapeutic levels of levonorgestrel detected in blood plasma of fish: results from screening rainbow trout exposed to treated sewage effluents

Jerker Fick, Richard H Lindberg, Jari Parkkonen, Björn Arvidsson, Mats Tysklind, D G Joakim Larsson

Supporting Information (8 pages)

Table S1. Information about the sewage treatment plants (STPs)

City	Inhabitants[a] $\times 10^3$	PE[b] $\times 10^3$	Flow[c] 2007 $Mm^3 y^{-1}$	Sludge[d] $Kton\ y^{-1}$	Hyd. t_r[e] h	Sol. t_r[f] D
Stockholm	710	850	86.5	15	24	15
Gothenburg	630	830	130	14	11	22
Umeå	90	110	13	2.7	8	20

[a] Inhabitants in the catchment area of each STP.
[b] Personal equivalents (average load from one person).
[c] raw sewage water flow.
[d] yearly production of digested dewatered sludge, dry weight.
[e] Retention time, hydraulic flow.
[f] Retention time in digester, solid flow.

Table S2. LogP and human therapeutic plasma concentrations for the included pharmaceuticals

	LogP [a]	HTPC [b] ($ng\ mL^{-1}$)	HTPC [b] references
Bezafibrate	4.3	15000	(Schulz and Schmoldt 2003) [c]
Carbamazepine	2.2	2000	(Schulz and Schmoldt 2003) [c]
Cilazapril	2.3	3	(Schulz and Schmoldt 2003) [c]
Clomipramine	5.7	20	(Schulz and Schmoldt 2003) [c]
Diclofenac	3.9	500	(Schulz and Schmoldt 2003) [c]
Diltiazem	2.8	20	(Schulz and Schmoldt 2003) [c]
Fexofenadine	2.8	300	(Schulz and Schmoldt 2003) [c]
Fluoxetin	4.6	160	(Schulz and Schmoldt 2003) [c]
Haloperidol	4.2	5	(Schulz and Schmoldt 2003) [c]
Ibuprofen	3.8	15000	(Schulz and Schmoldt 2003) [c]
Ketoconazole	4.4	1000	(Schulz and Schmoldt 2003) [c]
Ketoprofen	3.0	1000	(Schulz and Schmoldt 2003) [c]
Levonorgestrel	3.5	2.4	(Endrikat et al. 2002) [d]
Meclozine	5.9	10	(Park et al. 1977) [e]
Megesterol	4.0	10	(Miller et al. 1988) [f]
Memantine	3.3	80	(Periclou et al. 2006) [g]
Mianserin	3.4	10	(Schulz and Schmoldt 2003) [c]
Naproxen	3.1	20000	(Schulz and Schmoldt 2003) [c]
Orphenadrine	3.7	100	(Schulz and Schmoldt 2003) [c]
Oxazepam	2.3	200	(Schulz and Schmoldt 2003) [c]
Risperidone	3.5	6	(Schulz and Schmoldt 2003) [c]
Sertraline	5.3	50	(Schulz and Schmoldt 2003) [c]
Telmisartan	8.4	37	(Young et al. 2000) [h]
Tramadol	3.0	100	(Schulz and Schmoldt 2003) [c]
Verapamil	3.4	10	(Schulz and Schmoldt 2003) [c]

[a] Estimated log *P* values retrieved from EPI Suite™ KowWin

[b] Human therapeutic plasma concentration.

c Schulz M, Schmoldt A. 2003. Therapeutic and toxic blood concentrations of more than 800 drugs and other xenobiotics. Pharmazie 58:447-474.

[d] Endrikat J, Blode H, Gerlinger C, Rosenbaum P, Kuhnz W. 2002. A pharmacokinetic study with a low-dose oral contraceptive containing 20 mu g ethinylestradiol plus 100 mu g levonorgestrel. European Journal of Contraception and Reproductive Health Care 7:79-90.

[e] Park J, Logan R, Pottage A. 1977. Drug-Induced Extrapyramidal Signs in Chronic Liver-Disease - Case-Report. Clinical Toxicology 11:117-120.

[f] Miller AA, Becher R, Schmidt CG. 1988. Plasma concentrations of medroxyprogesterone acetate and megesterol acetate during long-term follow-up in patients treated for metastatic breast cancer. J Cancer Res Clin Oncol 114:186-190.

[g] Periclou A, Ventura D, Rao N, Abramowitz W. 2006. Pharmacokinetic study of memantine in healthy and renally impaired subjects. Clinical Pharmacology & Therapeutics 79:134-143.

[h] Young CL, Dias VC, Stangier J. 2000. Multiple-dose pharmacokinetics of telmisartan and of hydrochlorothiazide following concurrent administration in healthy subjects. J Clin Pharmacol 40:1323-1330.

Table S3. Liquid Chromatography – Electro Spray Ionization-Mass Spectrometry/Mass Spectrometry parameters.

Name	PREI [a]	PROI [b]	CE [c]	Rt [d]	LoQ [e]	LoQ [f]
	(m/z)	(m/z)	(a.u) [g]	(min)	(ng mL^{-1})	(ng L^{-1})
Bezafibrate	362.2	139.1	30	25.6	0.05	0.5
Carbamazepine	237.2	194.1	32	21.1	0.05	0.5
Cilazapril	418.2	211.1	25	18.1	0.1	1
Clomipramine	315.2	270.1	30	20.0	5	50
Diltiazem	415.2	178.1	35	17.7	0.5	5
Fexofenadine	502.2	171.1	50	19.2	0.5	5
Fluoxetine	310.2	148.1	25	19.3	1	10
Haloperidol	376.2	165.1	35	17.8	0.5	5
Ketoconazole	531.1	488.3	65	18.3	5	50
Levonorgestrel	313.2	109.1	30	28.6	0.1	1
Meclozine	391.2	201.1	25	22.5	0.05	0.5
Megestrol	385.2	224.1	30	30.0	5	50
Memantine	180.3	163.2	35	15.1	0.5	5
Mianserin	265.2	208.1	40	17.2	0.5	5
Orphenadrine	270.2	181.1	18	17.8	0.5	5
Oxazepam	287.2	241.1	30	21.9	0.5	5
Risperidone	411.2	191.1	40	14.8	0.5	5
Sertraline	306.2	275.1	20	19.5	0.5	5
Telmisartan	515.3	497.1	70	19.1	1	10
Verapamil	455.2	165.1	45	18.8	0.1	1

The source voltage was maintained at a constant 6.0 kV and the heated capillary temperature set to 250 °C.

[a] Precursor ion
[b] Product ion,
[c] Collision energy
[d] Retention time,
[e] Limit of quantification in fish plasma
[f] Limit of quantification in sewage effluent
[g] Arbitrary units

Table S4. Gas Chromatography-High Resolution Mass Spectrometry parameters.

Name	QnI [a]	QlI [b]	Rt [c]	LoQ [d]	LoQ [e]
	(m/z)	(m/z)	(min)	(ng mL^{-1})	(ng L^{-1})
Diclofenac	367.056	352.033	14.6	0.1	1
Ibuprofen	263.147	237.170	6.9	0.05	0.5
Ketoprofen	311.110		13.3	0.1	1
Naproxen	302.134	287.110	11.6	0.1	1
Tramadol	335.281	320.205	10.5	0.1	1

[a] Quantification ion
[b] Qualification ion
[c] Retention time
[d] Limit of quantification in fish plasma
[e] Limit of quantification in sewage effluent

Table S5. Absolute recoveries for the included pharmaceuticals.

	Fish plasma		Sewage effluent	
	%	SD	%	SD
Bezafibrate	154	13	126	1.1
Carbamazepine	109	5.8	123	4.1
Cilazapril	68	8.4	73	7.6
Clomipramine	87	9.0	43	6.9
Diclofenak	76	21	130	43
Diltiazem	89	9.2	63	17
Fexofenadine	78	9.2	56	6.9
Fluoxetin	104	8.5	102	14
Haloperidol	95	8.8	42	12
Ibuprofen	97	13	109	4.0
Ketoconazole	83	9.5	83	8.3
Ketoprofen	137	19	126	15
Levonorgestrel	105	13	92	7.4
Meclozine	133	6.8	37	9.2
Megestrol	105	10	98	6.5
Memantine	89	18	83	18
Mianserine	76	13	46	22
Naproxen	92	12	86	8.5
Orphenadrine	81	7.0	54	9.3
Oxazepam	108	7.9	111	4.0
Risperidone	119	28	18	13
Sertraline	89	4.2	39	8.2
Telmisartan	62	6.2	26	9.4
Tramadol	130	43	124	17
Verapamil	83	11	43	7.3

Figure S1. Chromatogram of levonorgestrel in fish plasma.