Determination of 17α-ethynylestradiol, carbamazepine, diazepam, simvastatin, and oxybenzone in fish livers

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Abstract

A method using liquid chromatography/tandem mass spectrometry (LC/MS/MS) was developed for the determination of 17α-ethynylestradiol in fish liver; a second method using LC/MS was developed for the determination of carbamazepine, diazepam, simvastatin, and oxybenzone in fish liver. The fish liver samples were extracted and cleaned up by using liquid-liquid extraction and solid-phase extraction before the extracts were analyzed by LC/MS or LC/MS/MS with electrospray negative and positive ionization. Recoveries of the five target compounds from spiked catfish liver ranged between 72 ±2% and 100 ±3%. Quantification limits for the five compounds ranged between 4.2 and 12.3 ng/g (wet weight). Ten turbot (*Pleuronichthys verticalis*) liver samples were analyzed; levels of 17α-ethynylestradiol, carbamazepine, simvastatin, and oxybenzone were below the detection limits. Diazepam was detected in all 10 fish liver samples at concentrations ranging between 23 and 110 ng/g (wet weight).

Introduction

Chemicals representing active ingredients in pharmaceuticals and personal care products (PPCPs) have recently emerged as environmental contaminants with adverse hormonal and toxic impacts on various organisms in the environment (Daughton and Ternes 1999, Ternes *et al*. 1999, Gomes *et al*. 2004, Noppe *et al*. 2007, Kim *et al*. 2007). A variety of PPCPs for human use are discharged, on a continual basis, into wastewater treatment plants (WWTPs) via excretion with urine and feces as parent compounds, conjugated compounds, or metabolites, and through washing or direct disposal (Daughton and Ternes 1999). At WWTPs, PPCPs can remain unchanged or undergo transformation during the treatment processes before being discharged into the environment via effluents and biosolids (Keller *et al*. 2003). Numerous PPCPs and their metabolites have been detected at concentrations as low as ng/L levels in a variety of environmental samples, including surface water, groundwater, drinking water, and sediment samples. Several review papers on analytical methods for PPCPs in water and sediment samples have been published (Golet *et al*. 2001, Öllers *et al*. 2001, Petrović *et al*. 2001, Sacher *et al*. 2001, Ternes 2001, Hyötyläinen and Hartonen 2002, Patterson *et al*. 2002, Simonich *et al*. 2002), but there is limited information on methods for the determination of PPCPs in such biological samples as fish tissues. Sensitive analytical methods for detecting PPCPs at trace levels in biological samples will help to better assess the impact of PPCPs on target organisms.

In this study, an LC/MS/MS method for detecting trace levels of 17α-ethynylestradiol in fish liver and an LC/MS method for carbamazepine, diazepam, simvastatin, and oxybenzone trace analysis in fish liver samples were developed. Background information for these five PPCPs is given in Table 1.

Methods

All reagents used in this study – acetonitrile, water, hexane, formic acid, methanol, and acetone – were LC grade, obtained from Fisher Scientific, Fair Lawn, NJ. Standards used in this study included 17α-ethynylestradiol, carbamazepine, diazepam, simvastatin, and oxybenzone (Sigma-Aldrich, St. Louis, MO), as well as stock and working standard solutions. Stock solutions of 17α-ethynylestradiol

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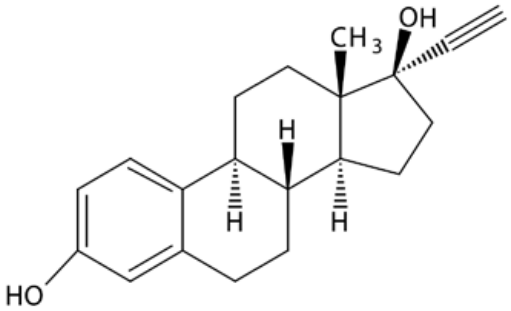
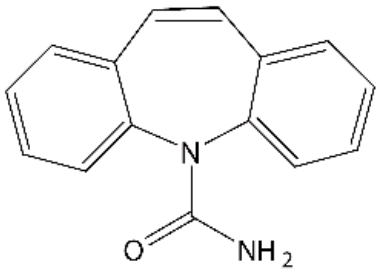
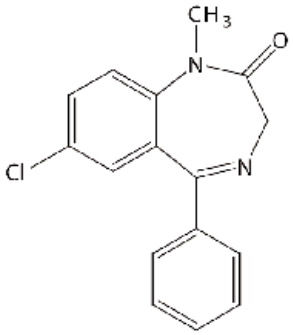
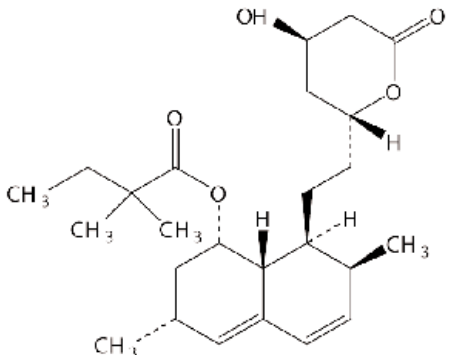
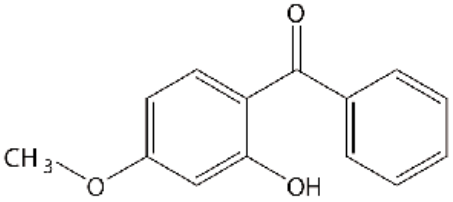
Table 1. Characteristics of compounds used in this study. Log K_{ow} calculated value found in SciFinder Database.

Compound	Log K_{ow}	Commercial Use	Chemical Structure
17α-Ethynylestradiol CAS # 57-63-6	4.15	Synthetic hormone in oral contraceptive pills	
Carbamazepine CAS # 298-46-4	2.8	Anticonvulsant and mood stabilizing drug, used primarily in the treatment of epilepsy and bipolar disorder	
Diazepam CAS # 439-14-5	3.0	Commonly used for treating anxiety, insomnia, seizures, alcohol withdrawal, and muscle spasms	
Simvastatin CAS # 79902-63-9	3.8	Lipid-lowering agent to control elevated cholesterol levels and to prevent cardiovascular disease	
Oxybenzone CAS # 131-57-7	3.4	Used in sunscreen for absorbing UV-A light	

and the other four PPCP compounds were prepared in methanol and acetonitrile, respectively, at a concentration of 1000 mg/L. For spiking and calibration, the 1000 mg/L stock solutions were serially diluted using either methanol or acetonitrile. Catfish liver purchased from a catfish processing plant was used for method development and recovery tests. The method was applied to the determination of the analytes of interest in 10 liver samples from turbot (*Pleuronichthys verticalis*) collected from southern California coastal waters.

Apparatus and respective sources for this study were as follows:

- Centrifuge: HN SII (Thermo IEC, Needham Heights, MA)
- Rotary Vacuum Evaporator: Rotavapor RE 121 (Büchi, Schweiz, Switzerland)
- Solid-phase Extraction (SPE) Columns: BAKERBOND SPE octadecyl C18, 1000 mg, 6 ml (J.T. Baker, Phillipsburg, NJ); Oasis HLB SPE, 500 mg, 6 ml (Waters Corp., Milford, MA)
- Polytetrafluoroethylene (PTFE) Filter: Millex-FH (Millipore, Bedford, MA)
- Homogenizer: Polytron PT 30-35 (Kinematica AG, Littau, Switzerland)
- Accelerated Solvent Extractor (ASE): ASE 200 (Dionex, Sunnyvale, CA)
- LC System: Waters Alliance 2695 Separations Module (Waters, Milford, MA) equipped with a quaternary, low-pressure mixing pump, inline vacuum degasser, and photodiode array detector with a wavelength range of 190-800 nm
- MS Detector: Waters Quattro-Micro Mass spectrometer (Micromass, Manchester, UK), a triple quadrupole mass spectrometer, equipped with an electrospray ionization source
- Analytical Column: Alltima reversed-phase column (250 x 2.1 mm, 5 µm particle size; Alltech, Deerfield, IL) for four PPCP compounds; and Alltima reversed-phase column (250 x 4.6 mm, 5 µm particle size; Alltech) for 17α-ethynylestradiol

Sample Extraction and Cleanup

Carbamazepine, diazepam, simvastatin, and oxybenzone in fish liver

An extraction method by Sanches-Silva *et al*. (2005) for detecting triclosan in chicken breast meat and Gouda cheese was slightly modified. A 2-g sample of fish liver was mixed with 10 ml of hexane; the mixture was shaken vigorously by hand for 10 minutes and centrifuged at 2054 x *g* for 10 minutes. Preliminary investigation showed that recoveries obtained by using 10 ml of hexane as the extractant were as good as those obtained using the acetonitrile and hexane mixture (1 + 5, v/v) proposed by Sanches-Silva *et al*. (2005). The supernatant was collected in a glass vial. The shaking and centrifugation procedure described above was repeated once with the residue in the centrifuge tube; the supernatant was then combined with that from the previous extraction. The combined supernatants were evaporated to dryness using a gentle nitrogen stream. The residue was dissolved in 2 ml of 90% acetonitrile in water using sonication; the resulting solution was diluted with 50 ml of water for cleanup by SPE, instead of filtration using filter papers as suggested by Sanches-Silva *et al*. (2005). The sample was passed through a SPE cartridge (Oasis HLB), preconditioned with 5 ml of methanol and then 5 ml of water, at 7 ml/minute. Subsequently, the cartridge was dried completely for 30 minutes, and the analytes were eluted twice with 5 ml of methanol. The methanol extracts were evaporated to dryness using a gentle nitrogen stream. The extract was dissolved in 4 ml of 90% acetonitrile in water using sonication; the solution was then filtered through a 0.45-µm PTFE filter before LC/MS analysis.

17α-ethynylestradiol in fish liver

This method was modified on the basis of a published paper by Shao *et al*. (2005). A 2-g sample of fish liver was extracted using 25 ml of water: methanol (1 + 4, v/v) and Polytron homogenization for 1 minute. The study's preliminary investigation showed better recoveries using a water:methanol mixture as the extractant than using methanol alone, as proposed by Shao *et al*. (2005). The homogenized sample was then centrifuged at 2054 x *g* for 10 minutes, and the supernatants were collected. The supernatants were transferred to a 250-ml separatory funnel and extracted with 20 ml of hexane twice to remove fat. The hexane layer was discarded. The aqueous phase (containing 3 ml of 1-propanol to pre-

vent bubble formation) was evaporated to approximately 5 ml in a rotary vacuum evaporator, then diluted with 50 ml of water. Instead of using an Oasis HLB SPE cartridge followed by a Sep-Pak silica-SPE cartridge for further sample cleanup, as proposed by Shao *et al*. (2005), this study used a BAKERBOND octadecyl C18 SPE cartridge followed by an Oasis HLB SPE cartridge for sample cleanup. For the BAKERBOND octadecyl C18 SPE cartridge, the diluted samples were introduced to the cartridge, which had been prewashed with 10 ml of methanol and 10 ml of water. After sample loading, the cartridges were allowed to dry under vacuum suction for 30 minutes. To collect 17α-ethynylestradiol, the cartridge was rinsed with 4 ml of acetonitrile:water (70 + 30, v/v). The elute was then diluted with 50 ml of water for further cleanup using an Oasis HLB SPE cartridge; the HLB cartridges had been preconditioned with 7 ml of methanol and 3 ml of water. After preconditioning, the samples were passed through the cartridges at 10 ml/minute. The cartridges were allowed to dry under vacuum suction for 30 minutes. The hormone was eluted with 5 ml of methanol twice. The eluted fractions were evaporated under nitrogen gas to dryness. Finally, the residue was reconstituted with 1 ml of methanol; the solution was then filtered through a 0.45-µm PTFE filter before LC/MS/MS analysis.

Recoveries from Spiked Samples

Analyte recovery was determined by spiking 2-g of catfish liver with a mixture of target compounds at concentrations of 10, 50, and 100 ng/g (wet weight) for each analyte. Those spiking concentrations were selected on the basis of the limit of quantification (LOQ), value determined for each analyte. The method described in the Code of Federal Regulations, Part 136, Appendix B (1993), suggests the spiking concentration for recovery tests should be at least one to five times higher than the LOQ. The spiked sample was homogenized and extracted; the extract was cleaned up by using the method described above and analyzed by using the following instrumental procedures. Intra-day recovery and precision tests were repeated six times for the analytes in the spiked samples. The inter-day recovery tests were repeated six times at two-day intervals for the spiked and real samples.

Instrumental Conditions

The LC/MS operating conditions for the five compounds of interest are listed in Tables 2 and 3. The MS data acquisition and analysis was performed using MassLynx Version 4.0 software.

Instrument Linearity and Range

Linearity was determined by constructing five calibration curves. For the construction of each calibration curve, 5 standard concentrations of 17α-ethynylestradiol, carbamazepine, diazepam, simvastatin, and oxybenzone in the range of 4 to 250 ng/ml were prepared in acetonitrile or methanol. Seven replicates of 25-µl injections were made for the standard solution to verify the repeatability of the detector response at each concentration. The peak areas of the chromatograms were plotted vs. the concentrations of the compounds of interest to obtain the calibration curves. The five concentrations of the standard solution were subjected to regression analysis by the least-squares method to calculate the calibration equation and correlation coefficient.

Identification and Quantification of Analytes

The structures of the five target compounds are shown in Table 1. The analytes in the samples were identified by comparing the chromatographic retention times and the LC/MS/MS spectra obtained for the samples with those of the standard compounds (Figure 1). The ion ratio must be within ±10% of the average of the reference standards in a given analytical run, as calculated by addition and subtraction. The ion pairs used for the ion at ratio calculation were ion at *m/z* 145/ion at *m/z* 184, ion at *m/z* 237/ion at *m/z* 194, ion at *m/z* 285/ion at *m/z* 287, ion at *m/z* 419/ion at *m/z* 285, and ion at *m/z* 229/ion at *m/z* 151 for 17α-ethynylestradiol, carbamazepine, diazepam, simvastatin, and oxybenzone, respectively. The retention time of the sample peak must match that of the average reference standard peak within ±0.25 minutes. 17α-ethynylestradiol was quantified by using LC/MS/MS to monitor the daughter ion at *m/z* 145 (Table 2). Carbamazepine, diazepam, simvastatin, and oxybenzone were quantified by using LC/MS to monitor their parent ions (Table 3). Calibration curves for quantification of target compounds in samples were obtained from external standards. The calibration curves were plots of the peak areas of target compounds vs. their concentrations in several calibration solutions.

Table 2. Operating conditions of liquid chromatography (LC) and mass spectrometry (MS) for 17α-ethynylestradiol LC/MS/MS analysis.

LC CONDITIONS	
Parameter	**Setting**
Column	Alltima reversed-phase column (250 x 4.6 mm, 5 µm particle size)
Mobile Phase	A: Water B: Acetonitrile
Solvent gradient	0 - 5 minutes 60% A; 5 - 15 minutes 60% A to 5% A; 15 - 17 minutes 5% A; 17 - 25 minutes 5% A to 60% A; 25 - 30 minutes 60% A
Flow Rate	1.0 ml/minute
Column Temperature	Ambient
Injection Volume	25 µl

MS CONDITIONS	
Parameter	**Setting**
Ionization Mode	Electrospray ionization, multiple reaction monitoring (MRM) in negative ion mode
Capillary Voltage	3.50 kV
Cone Voltage	70 V
Collision Energy	43 eV
Source Temperature	100 °C
Desolvation Temperature	300 °C
Cone Gas Flow	80 L/h
Desolvation Gas Flow	509 L/h
Collision Gas	Ar
Parent Ion (*m/z*)	295
Product Ion (*m/z*)	145

Determination of LOQ

The LOQ is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. The LOQ was determined on the basis of the standard deviation (sd) of the response and the slope (S) of the calibration curve according to the following formula: LOQ = 10 x sd/S (Code of Federal Regulations 1993). The sd was determined on the basis of the sd of the responses from seven matrix-matched standard solutions with the same lowest concentration of the calibration curve for the target analyte. The slope of the calibration curve was determined by the responses of matrix-matched standard solutions with seven different concentrations.

Results

Analyte Identification

The MS/MS spectra obtained for the target compounds with this system were similar to those found previously (Baronti *et al*. 2000, Fine *et al*. 2003, Miao and Metcalf 2003, van der Ven *et al*. 2004, Breton *et al*. 2005, Wang *et al*. 2005, Shimizu *et al*. 2007). The product ions and their percent relative abundances, compared with the abundance of the major product ion found for 17α-ethynylestradiol, were as follows: *m/z* 145 (100%), *m/z* 143 (44%), *m/z* 184 (41%), and *m/z* 295 (1%). The product ions and their percent relative abundances, compared with the abundance of the major product ion found for carbamazepine, were as follows: *m/z* 237 (100%),

Table 3. Operating conditions of liquid chromatography (LC) and mass spectrometry (MS) for carbamazepine, diazepam, simvastatin, and oxybenzone LC/MS analysis.

LC CONDITIONS

Parameter	Setting
Column	Alltima reversed-phase column (250 x 2.1 mm, 5 µm particle size)
Mobile Phase	A: Water with 0.1% formic acid B: Acetonitrile with 0.1% formic acid
Solvent Gradient	0 - 2 minutes 80% A; 2 - 15 minutes 80% A to 20% A; 15 - 25 minutes 20% A; 25 - 30 minutes 20% A to 80% A; 30 - 35 minutes 80% A
Flow Rate	0.3 ml/minutes
Column Temperature	Ambient
Injection Volume	25 ml

MS CONDITIONS

Parameter	Setting			
Ionization Mode	Electrospray ionization, selected ion recording (SIR) in positive ion mode			
Capillary Voltage	3.00 kV			
	Carbamazepine	Diazepam	Simvastatin	Oxybenzone
Cone Voltage	30 V	40 V	30 V	25 V
	Carbamazepine	Diazepam	Simvastatin	Oxybenzone
Collision Energy	20 eV	30 eV	30 eV	18 eV
Source Temperature	120°C			
Desolvation Temperature	300°C			
Cone Gas Flow	71 L/hour			
Desolvation Gas Flow	608 L/hour			
Collision Gas	Ar			
	Carbamazepine	Diazepam	Simvastatin	Oxybenzone
Parent Ion (*m/z*)	237	285	419	229

m/z 259 (37%), *m/z* 194 (8%), *m/z* 275 (4%), and *m/z* 138 (3%). The product ions and their percent relative abundances, compared with the abundance of the major product ion found for diazepam, were as follows: *m/z* 285 (100%), *m/z* 287 (41%), *m/z* 193 (1%), and *m/z* 154 (1%). The product ions and their percent relative abundances, compared with the abundance of the major product ion found for simvastatin, were as follows: (100%), *m/z* 419 (79%), *m/z* 338 (62%), *m/z* 199 (61%), and *m/z* 303 (49%). The product ions and their percent relative abundances, compared with the abundance of the major product ion found for oxybenzone, were as follows: *m/z* 229 (100%), *m/z* 151 (10%), *m/z* 338 (9%), and *m/z* 285 (7%).

The proposed fragments for some of the product ions of each compound are shown in Figure 1. For 17α-ethynylestradiol, a major daughter ion at *m/z* 145, $[M-H-150]^-$, is formed by fragmentation of the C-ring and formation of a double bond between the 9 and 8 positions (Fine *et al*. 2003). The ion at *m/z* 184 might be formed by loss of $C_7H_{11}O$. The mass spectrum of carbamazepine showed a daughter ion at *m/z* 194, $[M+H-44]^+$, formed by cleavage of the amide bond. The mass spectrum of diazepam exhibited daughter ion peaks at *m/z* 154, $[M+H-131]^+$, and *m/z* 193, $[M+H-92]^+$, formed by losses of C_8H_5NO and C_2H_3NOCl, respectively. The ion at *m/z* 287 is [M+H+2]+, reflecting the presence of one chlorine

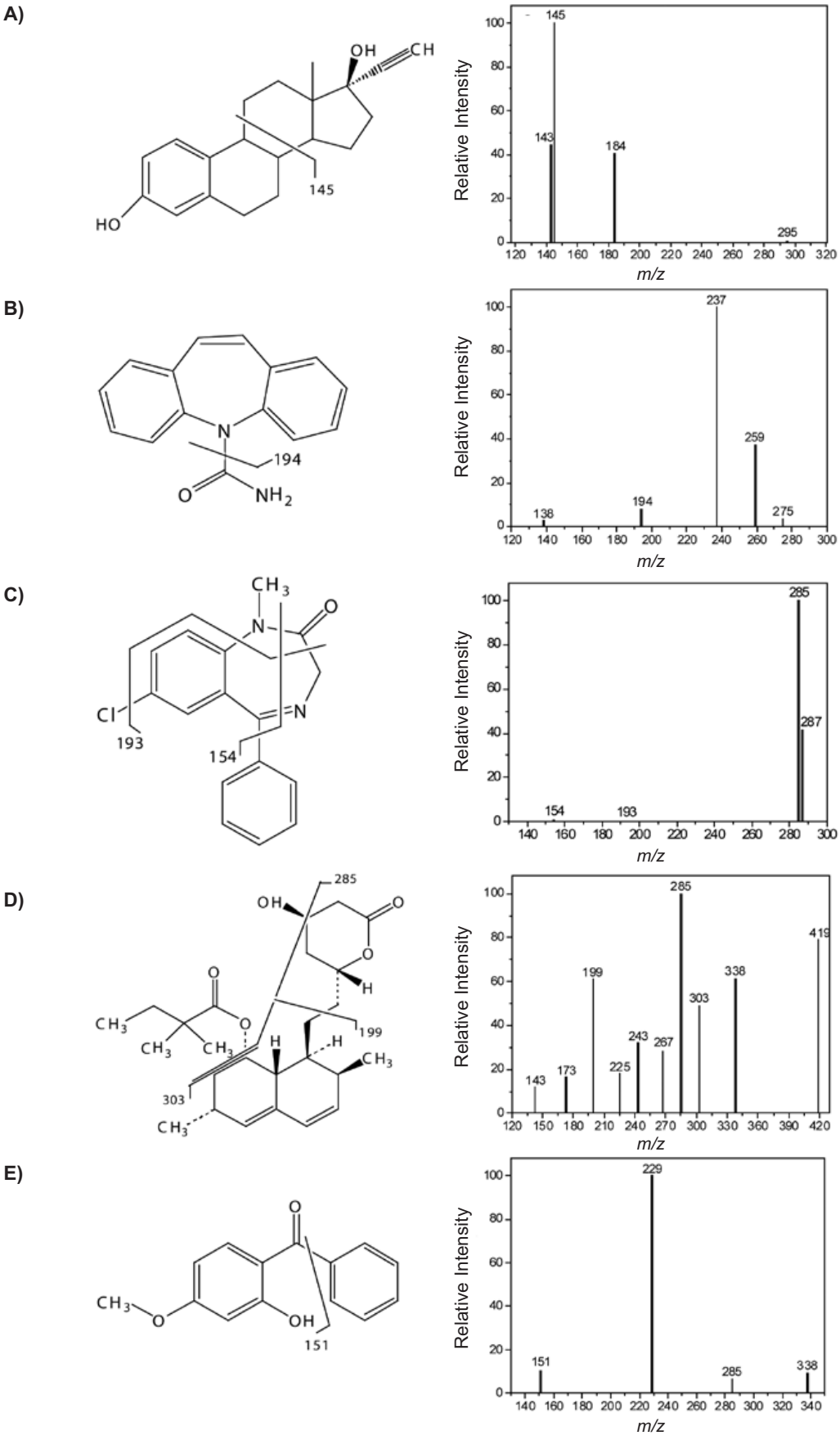


Figure 1. Experimental mass spectra of standards and proposed fragmentations for 17α-ethynylestradiol (A), carbamazepine (B), diazepam (C), simvastatin (D), and oxybenzone (E).

atom in the molecule. For simvastatin, a major fragment ion at *m/z* 285, $[M+H-134]^+$, was formed by loss of the ester side chain and a water molecule. The product ions at *m/z* 267, $[M+H-152]^+$, *m/z* 243, $[M+H-176]^+$, and *m/z* 199, $[M+H-220]^+$, were similar to those in mass spectra reported previously (Miao and Metcalf 2003, Wang *et al*. 2001). The ion at *m/z* 303 was also observed and formed by breakage of the ester side chain. The ion at *m/z* 285 was formed by the loss of one water molecule from the ion at *m/z* 303. The ion at *m/z* 267 was formed by the loss of one water molecule from the ion at *m/z* 285. The ion at *m/z* 243 was formed by the loss of CH_3COOH from the ion at *m/z* 285. The ion at *m/z* 199 was formed by the loss of C_2H_4O from the ion at *m/z* 243 (Wang *et al.* 2001). Oxybenzone showed a daughter ion at *m/z* 151, $[M+H-78]^+$, formed by the loss of a phenyl group.

Instrument Linearity and Range and Matrix Effect

The instrument calibration curves of the 5 target compounds were linear in the range of 4 to 250 ng/ml. All calibration curves used for quantification had r^2 values of 0.99. For the determination of 17α-ethynylestradiol in fish liver samples, ion suppression was found to be as high as 88% when only the BAKERBOND octadecyl C18 SPE column was used for cleanup of the extract. Ion suppression decreased to 23% with additional cleanup using an Oasis HLB SE column. When matrix-matched standards were used for the calibration curve, a 100 ±3% recovery rate was obtained for 17α-ethynylestradiol. No matrix effect was observed for the other four analytes.

Limit of Quantification

The LOQ value for 17α-ethynylestradiol in pure solution was 28% lower than that determined for the analyte in matrix-matched standards. No significant differences between LOQ values determined with pure standard solutions and matrix-matched standards were observed for the other four compounds because of the absence of matrix effect. As shown in Table 4, the matrix-matched LOQ values varied for the compounds of interest, ranging from 4.2 to 12.3 ng/g (wet weight), in fish liver.

Recovery and Precision

With the method described above, mean recoveries of the 5 target compounds added to catfish liver

Table 4. Analytical characteristics of the LC/MS methods developed for fish liver.

Compound	Spike Concentration (ng/g)	Recovery (% ± sd[a])	LOQ[b] (ng/g, wet weight)
17α-Ethynylestradiol	10	96 ± 8	12.3
	50	100 ± 3	
	100	98 ± 6	
Carbamazepine	10	79 ± 7	4.2
	50	74 ± 3	
	100	80 ± 3	
Diazepam	10	84 ± 4	8.2
	50	82 ± 10	
	100	86 ± 2	
Simvastatin	10	80 ± 7	7.9
	50	80 ± 4	
	100	80 ± 6	
Oxybenzone	10	73 ± 6	8.2
	50	72 ± 2	
	100	77 ± 5	

[a] Standard Deviation (n = 6)

[b] Matrix matched Limit of Quantification

at spiking levels of 10.0, 50.0, and 100.0 ng/g ranged from 72 ±2 to 100 ±3% (Table 4). Figures 2 and 3 show the chromatograms for the 5 target compounds added to catfish liver at 50.0 ng/g (wet weight).

The intra- and inter-day precision values were obtained with spiked samples. The ranges of the intra- and inter-day relative sd values for the 5 analytes determined in spiked samples using the above method were 2 to 8% and 2 to 13%, respectively. The inter-day precision values obtained with real samples were between 0.5 and 19%.

17α-Ethynylestradiol, Carbamazepine, Diazepam, Simvastatin, and Oxybenzone in Biological Samples

In order to confirm the LC/MS method developed for determination of the five PPCPs of interest in environmental samples, 10 marine fish liver samples were analyzed. The fish samples included five male and five female hornyhead turbot collected near ocean discharges of municipal wastewater effluent in southern California.

Diazepam was detected in all 10 fish liver samples at concentrations ranging from 23 to 45 ng/g (wet weight) in female fish livers and 58 to 110 ng/g (wet weight) in male fish livers of the same species. The calculations of the reported diazepam concentration levels in the fish liver samples were not corrected for the 82 ±10% to 86 ±2% recovery rates determined in this study. Figure 4 shows the LC/MS chromatogram of diazepam in one of the fish liver samples.

Discussion

To our knowledge, this article provides the first description and validation of methods for the determination of 17α-ethynylestradiol, diazepam, simvastatin, and oxybenzone in fish tissues. In one investigation, carbamazepine was determined in fillets of *Lepomis* sp. by using LC/MS with an LOQ of 0.12 ng/g (Ramirez *et al*. 2007). This LOQ is approximately an order of magnitude lower than the method described here, largely due to the difference in instrumentation.

This is the first report of the presence of diazepam in marine fish tissue. Diazepam is a lipophilic compound that ranked 34th among the top 300 prescriptions dispensed in the United States in 2005 (http://www.rxlist.com/script/main/art.asp?articlekey=7950). Diazepam has been reported to be present in municipal wastewater effluent at levels ranging from 0.02 to 6.3 µ/L (Andreozzi *et al*. 2003, Gómez *et al*. 2006, Vieno *et al*. 2006) and in river and drinking water at about 10 ng/L (Kolpin *et al*. 2002). Unpublished SCCWRP research results indicate diazepam is present at 0.001 to 0.008 µg/L in southern California municipal wastewater effluents discharged into coastal waters.

In our investigation, 17α-ethynylestradiol, carbamazepine, simvastatin, and oxybenzone were below their detection limits in the 10 fish liver samples (Figures 5 and 6). 17α-ethynylestradiol was recently reported to be present in ocean sediment at a level of <0.05 to 0.5 ng/g near sewage effluent in Australia (Braga *et al*. 2005). However, no previous report has been found on its levels in biological tissues.

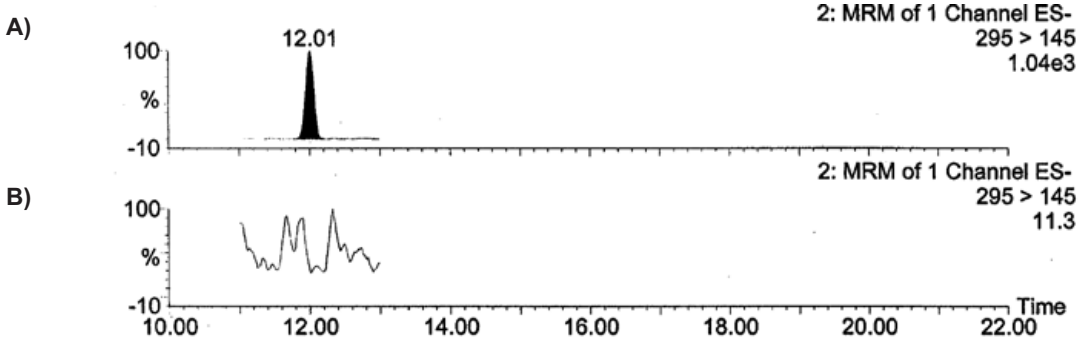


Figure 2. LC-MS/MS MRM chromatograms for 17α-ethynylestradiol that was spiked at 50 ng/g (wet weight) in catfish liver (A) and blank catfish liver (B). Y axis indicates peak height relative to the highest peak in each chromatogram. X axis indicates retention time. The numbers in the 2nd and 3rd line of the text at the upper right corner of each chromatogram indicates the *m/z* used for quantification, and peak area of target analyte, respectively.

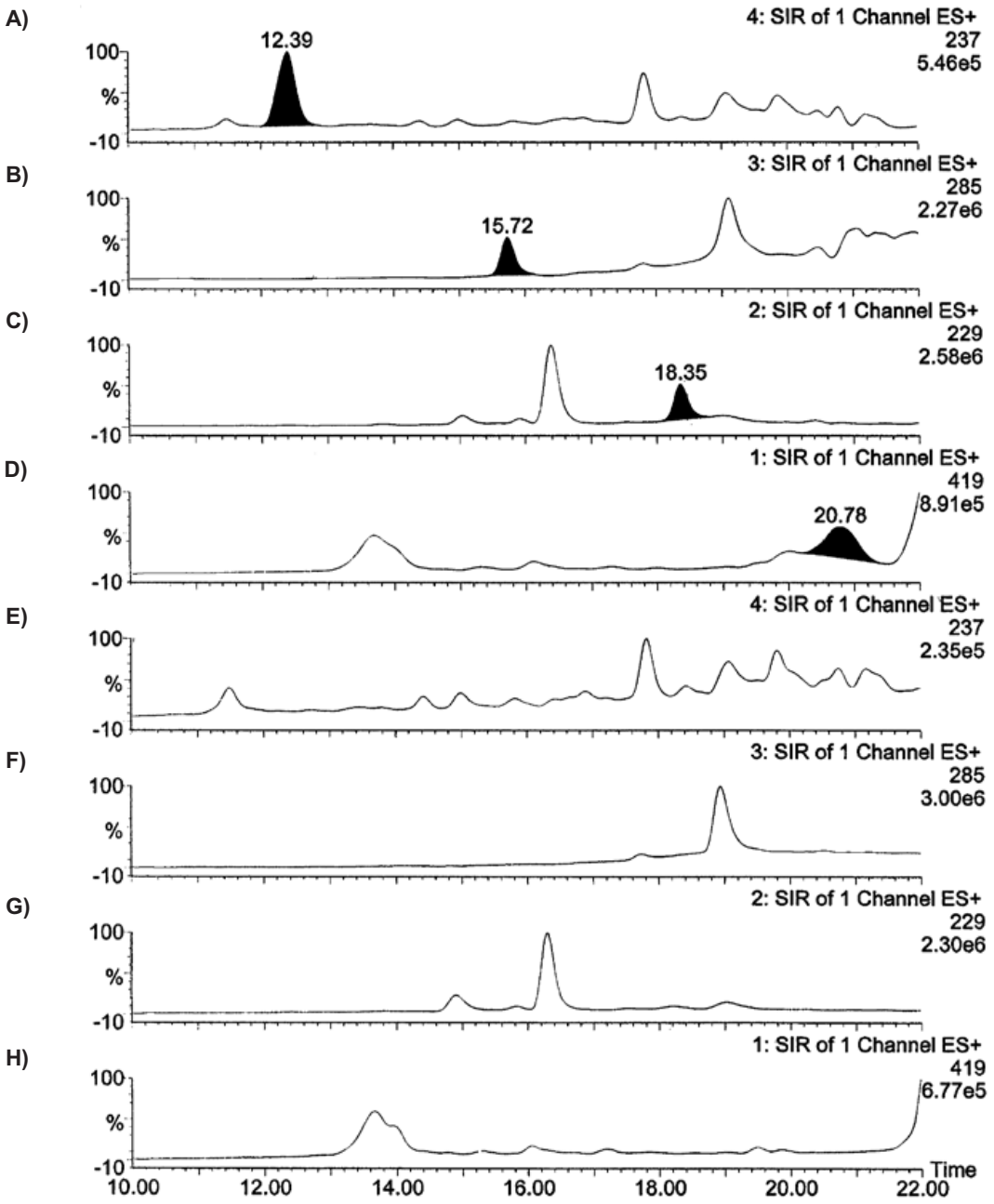


Figure 3. LC-MS SIR chromatograms for carbamazepine (A), diazepam (B), oxybenzone (C), and simvastatin (D) that were spiked at 50 ng/g (wet weight) in catfish liver. The blank catfish liver shows no detectable levels of the above listed compounds (E, F, G, and H). Y axis indicates peak height relative to the highest peak in each chromatogram. X axis indicates retention time. The numbers in the 2nd and 3rd line of the text at the upper right corner of each chromatogram indicates the *m/z* used for quantification, and peak area of target analyte, respectively.

Carbamazepine, simvastatin, and oxybenzone have been previously detected at low ng/L levels in wastewater samples (Vieno *et al*. 2006). In one report, carbamazepine was detected in *Lepomis* sp. fillets at concentrations ranging from 0.83 to 1.44 ng/g (wet weight; Ramirez *et al*. 2007). Concentrations of simvastatin and oxybenzone have not been reported in biological samples.

The tissue analysis methods described in this report provide a tool for investigating the fate of pharmaceuticals in aquatic environments and determining exposure levels in biota. Such information is essential for evaluating the relative risk of the many contaminants of emerging concern discharged into coastal and inland waters for which limited data on exposure and effects exist.

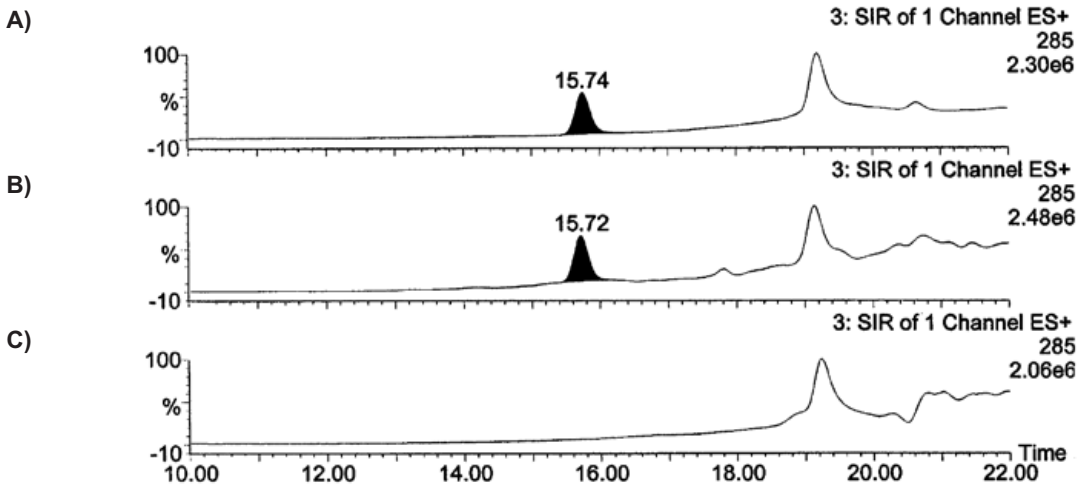


Figure 4. LC-MS SIR chromatograms of diazepam standard (A), diazepam in one of the turbot liver samples (B), and blank catfish liver (C). Y axis indicates peak height relative to the highest peak in each chromatogram. X axis indicates retention time. The numbers in the 2nd and 3rd line of the text at the upper right corner of each chromatogram indicates the *m/z* used for quantification, and peak area of target analyte, resepctively.

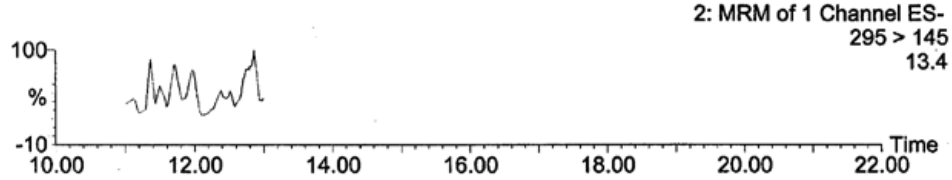


Figure 5. LC-MS/MS MRM chromatogram of 17α-ethynylestradiol in one fish sample showing no detectable level of 17α-ethynylestradiol. Y axis indicates peak height relative to the highest peak in each chromatogram. X axis indicates retention time. The numbers in the 2nd and 3rd line of the text at the upper right corner of each chromatogram indicates the *m/z* used for quantification, and peak area of target analyte, respectively.

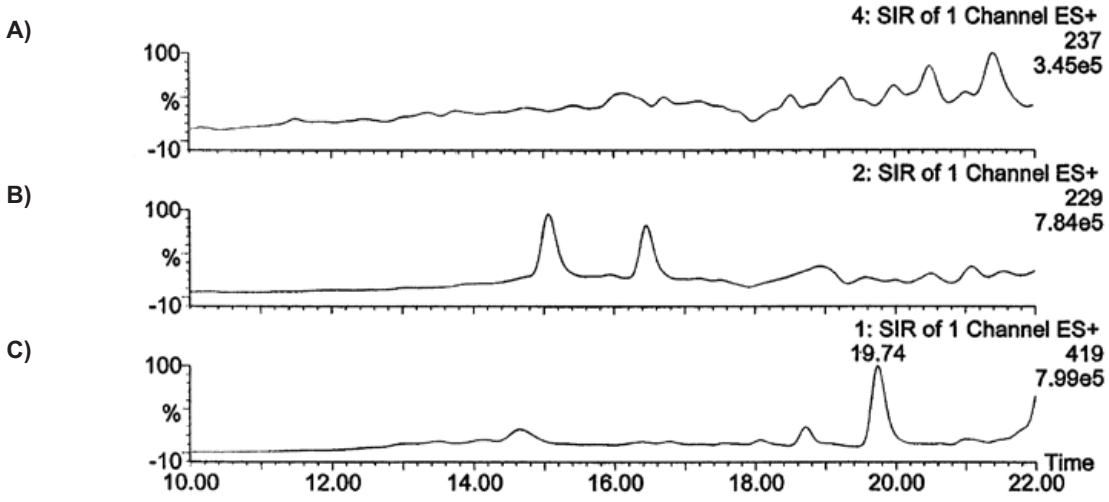


Figure 6. LC-MS SIR chromatogram for carbamazepine (A), oxybenzone (B), and simvastatin (C) in one fish sample showing no detection of the above listed target compounds. Y axis indicates peak height relative to the highest peak in each chromatogram. X axis indicates retention time. The numbers in the 2nd and 3rd line of the text at the upper right corner of each chromatogram indicates the *m/z* used for quantification, and peak area of target analyte, respectively.

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