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California GAMA Special Study: Analysis of Carbamazepine, Oxcarbazepine and Metabolites as Wastewater Tracers in Water Resource Studies

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GAMA: AMBIENT GROUNDWATER MONITORING & ASSESSMENT PROGRAM SPECIAL STUDY

California GAMA Special Study: Analysis of Carbamazepine, Oxcarbazepine and Metabolites as Wastewater Tracers in Water Resource Studies

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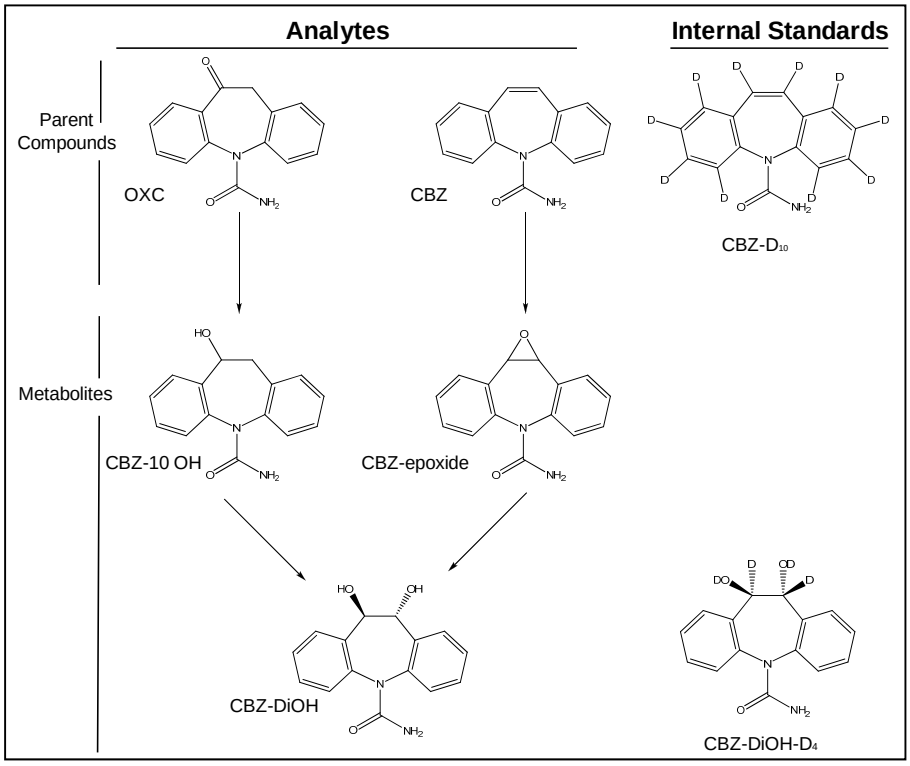

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Executive Summary

The Groundwater Ambient Monitoring and Assessment (GAMA) Program is a comprehensive groundwater quality monitoring program managed by the California State Water Resources Control Board (SWRCB). The GAMA Special Studies project provides analyses and interpretation of constituents of concern that allow assessment of current groundwater conditions. In addition, the Special Studies project develops analyses that will enhance the monitoring and assessment effort by focusing on specific constituents of concern and water quality parameters, such as disinfection byproducts (DBP), wastewater indicators, and redox conditions, as it relates to irrigation and groundwater management.

Wastewater indicators, i.e. chemical compounds that can be used to trace effluent from wastewater treatment plants or septic systems, are of considerable value for water management. Monitoring the fate and transport of these chemical tracers in WWTP effluents, surface waters, and groundwater can aid in the assessment of the degree of chemical contamination of water resources from various industrial, domestic, or husbandry sources. Such chemical tracers must be source-specific, appreciably soluble in water, and relatively resistant to biotic or abiotic degradation, including forced degradation during processes at a WWTP. The anti-epileptic/anti-convulsant drugs carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide) and oxcarbazepine (10,11-dihydro-10-oxo-5H-dibenz[b,f]azepine-5-carboxamide) are thought to be good potential tracers owing to satisfaction of such requirements (Leclercq et al., 2009).

Carbamazepine (CBZ; approved in 1974) and oxcarbazepine (OXC; approved in 2000) have become some of the most widely prescribed drugs for the treatment of epilepsy, trigeminal neuralgia, and bipolar disorder. In a study of pharmaceuticals in WWTP of Finland, CBZ was determined to be one of the most heavily used drugs, with a per capita consumption rate of 2.4 mg/day. It has been one of the more frequently identified pharmaceuticals in WWTP effluents and river water. Additionally, CBZ was one of eleven compounds most frequently detected in a survey study of 51 compounds in source water (found in 79% of samples), finished drinking water (44% of samples), and distribution system water (40% of samples) sampled from 19 U.S. facilities that provide water service for 28 million people.

Urinary metabolites of pharmaceuticals may also be good tracers of wastewater. Metabolism of CBZ, for example, is sufficiently efficient that only 1% of ingested CBZ is excreted unchanged in the urine. The primary urinary metabolites of CBZ are carbamazepine-epoxide (CBZ-E) and 10,11-dihydro-10,11-dihydroxycarbamazepine (CBZ-DiOH). The primary urinary metabolites of

OXC are 10,11-dihydro-10-hydroxycarbamazepine (CBZ-10-OH) and CBZ-DiOH. The CBZ-DiOH metabolite has been found in wastewater effluent and environmental samples at higher concentrations than the parent compounds. These parent drugs and their major metabolites may serve as useful tracers for municipal wastewaters and effluent impact on water resources given the pharmaceuticals' long use, high rate of consumption, and stability to both biotic and abiotic degradation processes.

This study developed a robust analytical method for the quantitation of CBZ, OXC, CBZ-E, CBZ-DiOH, and CBZ-10-OH in wastewater treatement plant (WWTP) effluent and in groundwater in the parts per trillion range. The method uses commercially available solid-phase extraction (SPE) cartridges and liquid chromatography tandem mass spectrometry (LC/MS/MS), with quantitation from a labeled internal standard. The limit of quantitation ranged from 0.01 to 0.1 ng on-column. Surveyed water samples included WWTP primary, secondary, and tertiary treatment samples. Concentrations of metabolites in effluent are detailed here for the first time for U.S.-based WWTP.

While such work as been reported before by other groups, the work presented here is novel for using an isotopically-labeled internal CBZ standard for quantitation and compensation for matrix effects as well as providing data for CBZ, OXC *and* metabolite concentrations at a U.S.-based WWTP. As discussed, quantitation of CBZ, OXC and the various metabolites as novel wastewater tracers may allow for the estimation of the contamination of surveyed subsurface water sites by domestic sources.

The main body of the report consists of a manuscript being prepared for publication in a peer-reviewed journal, and a protocol for sample preparation and analysis of wastewater and groundwater samples for determination of CBZ, OXC and their metabolites by SPE-LC/MS/MS.

Owens J. E., Vu A. K., and Esser B. K. (2010) Use of Carbamazepine, Oxcarbazepine and Metabolites as Novel Wastewater Tracers in Assessment of Contamination of Water Resources, ***in prep.***

Appendix A: Protocol for the Extraction, Clean-Up, and Analysis of Carbamazepine and Related Metabolites from Collected Water Samples by Solid Phase Extraction with Analysis by LC/MS/MS

-+Use of Carbamazepine, Oxcarbazepine and Metabolites as Novel Wastewater Tracers in the Assessment of Contamination of Water Resources

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ABSTRACT

Pharmaceuticals in the environment have attracted significant attention in recent years. The method here details the analysis of carbamazepine (CBZ), oxcarbazepine (OXC) and metabolites carbamazepine-epoxide (CBZ-epoxide), 10,11-dihydro-10-hydroxycarbamazepine (CBZ-10-OH), and 10,11-dihydro-10,11-hydroxycarbamazepine (CBZ-DiOH), from sampling sites within a wastewater treatment plant (WWTP). Samples (500 mL) were prepared using Oasis HLB solid phase extraction cartridges and analyzed by liquid chromatography tandem mass spectrometry and quantified using an internal standard (CBZ-D_{10}). The limit of quantitation ranged from 0.01 to 0.1 ng on-column. Composite samples were fortified with a mix of parent pharmaceuticals and metabolites for method validation. Reasonable recoveries were assessed for CBZ, CBZ-epoxide, and CBZ-10-OH, and CBZ-DiOH (> 70%) and low recoveries (44%) for OXC were observed. Concentrations of metabolites in effluent are detailed here for the first time for U.S.-based WWTP.

Keywords: carbamazepine, oxcarbazepine, metabolites, liquid chromatography, mass spectrometry

INTRODUCTION

Chemical compounds that can be used to trace effluent from wastewater treatment plants (WWTPs) are of considerable value for management of water resources. Monitoring the fate and transport of these chemical tracers in WWTP effluents, surface waters, and groundwater can aid in the assessment of the degree of chemical contamination of water resources from various industrial, domestic, or husbandry sources. Such chemical tracers must be source-specific, appreciably soluble in water, and relatively resistant to biotic or abiotic degradation, including forced degradation during processes at a WWTP. The anti-epileptic/anti-convulsant drugs carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide) and oxcarbazepine (10,11-dihydro-10-oxo-5H-dibenz[b,f]azepine-5-carboxamide) are thought to be good potential tracers owing to satisfaction of such requirements (Leclercq et al., 2009).

Carbamazepine (CBZ; approved in 1974) and oxcarbazepine (OXC; approved in 2000) have become some of the most widely prescribed drugs for the treatment of epilepsy, trigeminal neuralgia, and bipolar disorder. In a study of pharmaceuticals in WWTP of Finland, CBZ was determined to be one of the most heavily used drugs, with a per capita consumption rate of 2.4 mg/day (Vieno et al., 2007). It has been one of the more frequently identified pharmaceuticals in WWTP effluents and river water (Gagne et al., 2006; Miao and Metcalfe, 2003; Miao et al., 2005) and recent work aimed at elucidating the ecotoxicological effects of CBZ exposure on non-human organisms (Martin-Diaz et al., 2009). Additionally, CBZ was one of eleven compounds most frequently detected in a survey study of 51 compounds in source water (found in 79% of samples), finished drinking water (44% of samples), and distribution system water (40% of samples) sampled from 19 U.S. facilities that provide water service for 28 million people (Benotti et al., 2009). These parent drugs and their major metabolites (**Figure 1**), which

include carbamazepine-epoxide (CBZ-epoxide), 10,11-dihydro-10-hydroxycarbamazepine (CBZ-10-OH), and 10,11-dihydro-10,11-hydroxycarbamazepine (CBZ-DiOH) (Kupferberg, 1982), may serve as useful tracers for municipal wastewaters and effluent impact on water resources given the pharmaceuticals' long use, high rate of consumption, and stability to both biotic and abiotic degradation processes.

CBZ and its diol metabolites are unstable during analysis by GC (Baker et al., 1973; von Unruh and Paar, 1986) and derivatization techniques are occasionally employed to increase analyte stability (von Unruh and Paar, 1986). CBZ, OXC, and metabolites are now frequently analyzed using liquid chromatography with detection by mass spectrometry or tandem mass spectrometry (LC/MS or LC/MS/MS) for various matrices, including urine (Maggs et al., 1997), plasma (Breton et al., 2005; Corso et al., 2007; Maia et al., 2007) aqueous (Leclercq et al., 2009; Miao and Metcalfe, 2003) and bio-solid (Miao et al., 2005) samples. Various clean-up and extraction techniques have been employed, including liquid-liquid extraction using diethyl ether-methylene chloride (Maia et al., 2007), pressurized liquid extraction (Miao et al., 2005), on-line solid phase extraction (Trenholm et al., 2009) or solid phase extraction (SPE) with C8 phases (Corso et al., 2007) or Oasis HLB phases (Benotti et al., 2009; Leclercq et al., 2009; Miao and Metcalfe, 2003; Vanderford et al., 2003). Reported limits of detection (LOD) and limits of quantitation (LOQ) for these various matrices with LC/MS/MS analysis ranged from 0.4 ng/L (Miao and Metcalfe, 2003) to 500 ng/L (Breton et al., 2005) for CBZ, 20 ng/L (Maia et al., 2007) to 400 ng/L for OXC (Breton et al., 2005), and 0.1 ng/L (Miao and Metcalfe, 2003) to 300 ng/L for the metabolites (Breton et al., 2005).

The purpose of the work presented here was to develop and optimize an SPE method for the extraction of CBZ, OXC, and major metabolites, CBZ-epoxide, CBZ-10-OH and CBZ-DiOH

(**Figure 1**), from various sampled waters followed with analysis by LC/MS/MS at part-per-trillion (ng/L) levels. These surveyed water samples included WWTP primary, secondary, and tertiary treatment samples. While such work as been reported before by other groups (Kinney et al., 2006; Leclercq et al., 2009; Miao and Metcalfe, 2003; Miao et al., 2005; Trenholm et al., 2009), the work presented here is novel for using an isotopically-labeled internal CBZ standard for quantitation and compensation for matrix effects as well as providing data for CBZ, OXC *and* metabolite concentrations at a U.S.-based WWTP. As discussed, quantitation of CBZ, OXC and the various metabolites as novel wastewater tracers may allow for the estimation of the contamination of surveyed subsurface water sites by domestic sources.

MATERIALS AND METHODS

Units

Concentrations are expressed as parts per million (micrograms per milliliter or μg/mL) for primary standards; parts per billion (nanograms/milliliter or ng/mL) for secondary standards and for spiked samples; and as parts per trillion (nanograms/liter or ng/L) for un-spiked samples.

Chemicals and Reagents

CBZ and CBZ-epoxide were purchased from Sigma-Aldrich (St. Louis, MO) at a minimum of 98% purity. A ClinCal calibrator containing CBZ-DiOH (3600 ng/mL), CBZ-10-OH (27800 ng/mL) CBZ-epoxide (5600 ng/mL), OXC (20800 ng/mL), and CBZ (11000 ng/mL) was purchased from Recipe Chemicals + Instruments GmbH (Munich, Germany). The internal standard (IS) CBZ-D_{10} (> 98% purity) was from Cambridge Isotope Laboratories at a

concentration of 100 µg/mL in acetonitrile. HPLC-grade water (18 MΩ) was prepared using a Barnstead Nanopure filtration system. HPLC-grade solvents including methanol, acetonitrile, and ethyl acetate were from Fisher Scientific, as were all other chemicals unless specified otherwise.

Standard Preparation

Preparation of Internal Standard

The IS CBZ-D_{10} was purchased at a concentration of 100 µg/mL in acetonitrile. Fifty µL was dissolved in methanol to prepare 5 mL at 1 µg/mL. Ten µL was added to every water sample prior to extraction and clean-up by SPE.

Standards for the Optimization of SPE Protocol

CBZ from Sigma-Aldrich was prepared by weighing out approximately 30 mg and dissolving in 25 mL methanol to prepare a stock solution of 1195.2 µg/mL. CBZ-epoxide was prepared by dissolving approximately 3 mg in 25 mL methanol for a stock solution concentration of 105.8 µg/mL. Using these stock solutions, a *working stock solution I* in methanol was prepared that contained CBZ and CBZ-epoxide at a concentration of 10,000 ng/mL. Using the *working stock solution I*, calibration standards were prepared in methanol ranging from 0.5 ng/mL to 1,000 ng/mL.

Standards for Method Verification Procedures and Sampled Water Analyses

The ClinCal standard was reconstituted with 3 mL HPLC-grade water and 600 µL aliquots were pipetted into multiple amber glass autosampler vials to avoid multiple freeze/thaw cycles. Standards were prepared in HPLC-grade water ranging from 0.18 ng/mL to 2,780 ng/mL, with

specific concentrations for each analyte shown in **Table 1**. Ten µL of IS was added to each standard for a total final volume of 1 mL. When not in use, standards were stored at 4 °C.

Instrumental Conditions

A Waters Alliance HT 2795 liquid chromatograph with a Waters Micromass Quattro Micro *api* triple quadrupole mass spectrometer (Waters Corporation, Milford, MA) was used for analysis. The mass spectrometer was first calibrated using a solution of sodium iodide/cesium iodide (both purchased from Sigma-Aldrich) per manufacturer's specifications. Tune parameters for CBZ, CBZ-epoxide and IS (**Table 2**) were established by infusing an approximate 50 µg/mL solution of each prepared in methanol/water (50/50, v/v) containing 0.1% formic acid at 20 µL/min. A diluted volume of the ClinCal standard was infused to check masses and product ions of additional analytes OXC, CBZ-DiOH, and CBZ-10-OH. Settings were: capillary at 3.0 kV (positive ion mode), cone voltages were variable (**Table 2**), extractor at 2 V, RF lens at 0.2 V, source temperature at 120 °C, desolvation temperature at 300 °C, desolvation gas flow at 500 L/h, cone gas flow at 45 L/h, low mass and high mass resolution at 14.5, ion energy 1 at 0.5, entrance energy at -1 eV, collision energies were variable (**Table 2**), exit energy at 2 eV, low mass and high mass resolution 2 at 15.0, ion energy 2 at 1.5, and multiplier was set to 650 arbitrary units. The dwell time was 0.1 ms, inter-channel delay was 0.1 s, inter-scan delay was 0.1 s, repeats at 1, and the span set to 0 Da. All analytes and IS were analyzed using multiple reaction monitoring (MRM) and the transitions listed in **Table 2** were monitored.

The Waters 2795 HPLC consisted of a quaternary pump, in-line mobile phase degasser, temperature-controlled autosampler (maintained at 15 °C) and column compartment (maintained at 30 °C). Twenty µL of standard mix or prepared sample was injected onto a 150 x 2.1 mm i.d.,

3.5 µm particle XBridge C18 analytical column (Waters Corp.). Mobile phase A was water + 0.1% formic acid and mobile phase B was acetonitrile + 0.1% formic acid. The gradient program was as follows: at 0 min, 80% A (hold for 2 min) to 20% A at 8 min (hold for 2 min) returning to 80% A at 11 min (hold for 4 min) for a total run time of 15 min. The column was equilibrated for two minutes between samples. A constant flow rate of 0.2 mL/min was maintained throughout. CBZ-DiOH eluted at 5.35 min, CBZ-10-OH at 7.04 min, CBZ-epoxide at 7.94 min, OXC at 8.28 min, and CBZ and CBZ-D_{10} at 9.02 min (**Figure 2**).

Quantitation was by quadratic regression with $1/x$ weighting with $n \geq 2$ measurements per standard. A standard curve was prepared at the beginning of each sequence run and individual standards were included throughout the sequence list after every six matrix samples. The responses of these the check calibration standards were also included in the standard curve preparation. Example chromatograms of a standard at Level 6, water blank, and water sample are shown (**Figure 2**).

Sampling

Samples were collected in triplicate at three sampling stations within the Livermore Water Reclamation Plant (LWRP) located in Livermore, CA. Samples were collected of primary (at sedimentation tanks), secondary (post-chlorination), and tertiary (post-UV) effluent. At each sampling site, unfiltered samples were collected into two 1-L pre-cleaned I-CHEM (Thermo-Fisher Scientific, Rockwood, TN) amber glass bottles and one 1-L I-CHEM HDPE bottle. All samples were put on ice immediately after collection. Large-volume samples are stored in the refrigerator (4 °C) until sample preparation for LC/MS/MS analysis. Samples were prepared for analysis within 48 hours after sample collection.

Water Sample Preparation by Solid Phase Extraction

Five hundred mL of the collected water samples were prepared for LC/MS/MS analysis using Oasis HLB columns (150 mg/6 cc; Waters Corp.). Ten µL of IS was added to the 500 mL of water sample prior to clean-up and extraction by SPE. The columns were conditioned with two column volumes of methanol followed by two column volumes of HPLC-grade water. The collected water samples were applied to the conditioned columns using a peristaltic pump such that the flow rate was 0.6 mL/min, for an approximate loading time of 14 hours. The columns were washed with 2 mL HPLC-grade water and eluted with 0.5 mL methanol followed by 0.5 mL ethyl acetate. Two hundred µL were transferred to a secondary vial and evaporated to near dryness using ultra-pure nitrogen. The samples were re-constituted in 75 µL HPLC-grade water and transferred to a PolySpring 100 µL insert. The samples were stored at 4 °C until analysis.

Method Verification

A composite matrix consisting of equal parts volume of sampled water collected at the three sampling locations at the LWRP was used for method verification studies. Three 125 mL aliquots of the composite matrix were each spiked with 25 µL of the Level 1 calibration standard that did not contain IS. A second sample set was not spiked and served as a control group. Additionally, 10 µL of the IS mix containing CBZ-D_{10} was added to samples in both sets. The spiked and control sample sets were then prepared for analysis using the optimized solid phase extraction protocol described above. The samples were stored at 4 °C until analysis.

Holding Time Study

Six aliquots of 125 mL were taken of the effluents from each of the three sampling stations (n=18) and subsequently spiked with 100 µL of the Level 1 calibration standard (did not contain IS). All samples were mixed well and one set of three samples from each study area was prepared by SPE and LC/MS/MS analysis as described immediately after mixing. The second set of three spiked samples from each sampling area was stored at 4˚C for one week prior to sample preparation and analysis by LC/MS/MS as described above.

Statistical Analyses

All statistical analyses were completed using Analysis ToolPak from Microsoft Excel. Such analyses included a two-tailed Student's *t*-test assuming equal variances and single-factor analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Method Characteristics

The quadratic regression (with $1/x$ weighting) of the internally-standardized calibration curves had minimum R^2 values of 0.9913. The check calibration standards that were analyzed after every six samples were included in the standard curve preparation. The limits of detection (LODs) were determined by calculating the peak-to-peak signal to noise (S/N = 3) for the confirmation ion. The limits of quantitation (LOQs) were determined by calculating the S/N (where S/N = 10) for the quantitation ion (**Table 3**).

To determine the intra-day variability for the standard and water sample, samples were repeatedly analyzed (n = 3) within one day. The variability as shown by the relative standard deviation (RSD, %) was 19.4% or less for analytes in the standard, and 25.0% or less for those same analytes extracted from a spiked water sample by SPE. Inter-day variability for the standards was 15.6% or less (**Table 3**).

Method Verification

Composite samples of LWRP primary, secondary, and tertiary effluent were run with and without spiking (**Table 4**). Un-spiked control samples (n = 3) were prepared for analysis using the optimized SPE method. In these samples, all analytes were detected, though concentrations of OXC were below the LOQ (4.2 ng/mL). The concentrations of CBZ-epoxide were just at the LOQ and resulting precision was poor (RSD of 37.5%). All other analytes (CBZ, CBZ-10-OH, and CBZ-DiOH) were found in appreciable quantities ($\geq$ 29.3 ng/mL).

Spiked samples were prepared by adding 25 µL of a Level 1 calibration standard that did not contain IS to aliquots of the composite samples. The spike concentrations per 125 mL composite sample ranged from 9 ng/mL (CBZ-DiOH) to 69.5 ng/mL (CBZ-10-OH). Spiked composite samples were also cleaned up prior to analysis using the SPE method. Resulting recoveries ranged from 44% (OXC) to 105% (CBZ) with reasonable precision (< 25%) with the exception of CBZ-DiOH (57%).

Optimization of Solid Phase Extraction Methodology

Six different SPE columns were evaluated to optimize the efficient recovery of the analytes CBZ and CBZ-epoxide in a 500 ng/mL standard prepared in reagent water. The following

columns were evaluated: Alltech C8 (200 mg/4 cc; Deerfield, IL), Supelco Discovery DSC-18 (500 mg/6 cc; Bellefonte, PA), Phenomenex strata-X (30 mg/1 cc; Torrance, CA), and Waters Oasis HLB (200 mg/5 cc glass cartridge; 150 mg/6cc, and 60 mg/3 cc). All columns were conditioned, loaded, washed, and eluted using solvents and solvent volumes recommended per manufacturer's specifications. The resulting recoveries of CBZ and CBZ-epoxide were 93 – 105% by external calibration and 104 – 110% by internal calibration when using the DSC-18, Alltech C8, and strata-X, and Oasis HLB 60 mg columns. Use of the Oasis HLB 150 mg columns resulted in recoveries of approximately 70% for CBZ and 75% for CBZ-E using either internal or external calibration methods. Recoveries by the glass Oasis HLB columns (200 mg/5 cc) resulted in approximately 30 – 37% for CBZ and CBZ-epoxide with RSD of 49 – 56%.

The optimal peak shape was observed when the Oasis HLB 60 mg columns or the Supelco DSC-18 columns were used for sample preparation and clean-up. To optimize the SPE method using one of these columns for large sample volumes, samples of reagent water (400 mL) were spiked with 50 µL each with the reconstituted ClinCal standard containing all analytes of interest (including CBZ, OXC, and metabolites). The samples were applied to the SPE columns at 0.6 mL/min through either the conditioned Oasis HLB 60 mg columns or Supelco DSC-18 columns by using a peristaltic pump as previously described. The columns were washed with 1 column volume of water and the analytes were eluted with 1 mL methanol into an amber glass autosampler vial. Ten µL of IS was added and mixed well. Two hundred µL aliquots were transferred to a clear autosampler vial and gently evaporated to dryness using ultra-high purity nitrogen. The samples were re-constituted in 100 µL of water and placed into an autosampler vial insert prior to analysis by LC/MS/MS. Improved peak shape and optimized resolution between CBZ-epoxide and OXC were observed when samples were prepared in HPLC water as

opposed to a solvent or solvent mix. Use of the Oasis HLB 60 mg columns resulted in better recoveries for all analytes compared to the Supelco DSC-18 columns, with 73% recovery for CBZ, 70% recovery for OXC, 67% recovery for CBZ-epoxide, 42% recovery for CBZ-10-OH, and 47% recovery of CBZ-DiOH from the larger volumes of reagent water (400 mL).

However, the recoveries of the more polar metabolites (CBZ-10-OH and CBZ-DiOH) were low when using the Oasis HLB 60 mg columns. In a follow-up experiment, the Oasis HLB 150 mg columns were then re-evaluated as it was hypothesized that the increased sorbent mass would result in increased recoveries of the more polar metabolites when cleaning up large volumes of water. In this experiment, 10 µL of the ClinCal standard mix was spiked into 1 L HPLC grade water. The spiked water samples were loaded onto conditioned Oasis HLB 60 mg and 150 mg columns (n = 3 each) at 0.6 mL/min by peristaltic pump as described. The columns were washed with reagent water and eluted with 0.5 mL methanol followed by 0.5 mL ethyl acetate. Again, 10 µL IS was added prior to mixing and evaporation of a 200 µL aliquot. While the differences in recoveries for the different analytes between the two columns were not statistically significant ($P \geq 0.1856$ by two-sided Student's *t*-test assuming equal variances), the recoveries of the analytes tended to be more precise (RSD of 11% or less) when using the Oasis 150 mg columns. With these columns, the absolute recovery of CBZ from 1 L of water was 86%, OXC at 97%, CBZ-epoxide at 82%, CBZ-10-OH at 51%, and CBZ-DiOH at 59%. The 10 µL of IS was then added to the collected 1 L sample prior to SPE clean-up to correct for any losses of analytes.

Quantification of CBZ, OXC, and Metabolites in Wastewater

Samples of primary (before secondary treatment), secondary (after chlorination), and tertiary (after UV-treatment) wastewater were collected from the LWRP. The samples were analyzed in

triplicate and results of analyses (mean concentrations with % RSD) are shown in **Table 5**. The precision of the analyses, as indicated by %RSD, were good for these collected environmental samples. At the primary stage of the LWRP, the maximum RSD was 15% for CBZ-epoxide, which had a mean concentration of 8.3 ng/L. Concentrations of the compounds ranged from below the LOQ (for oxcarbazepine) to 392 ng/L for CBZ-DiOH. At the secondary treatment stage, the %RSD was 26% (for CBZ) or lower for the five analytes. Concentrations were generally higher (with the exception of CBZ-DiOH) at the secondary stage, which has been previously observed (Leclercq et al., 2009). A lower concentration of CBZ-DiOH between the primary and secondary stages might indicate that this compound is somewhat susceptible to degradation by chlorination, though Benotti and colleagues have indicated that CBZ degradation by chlorinating processes is pH-dependent and is measureable when WWTPs use chlorination processes for disinfection over ozone treatment (Benotti et al., 2009). Recent work by Rosal et al. showed that carbamazepine required ~ 130 μM ozone for degradation (Rosal et al., 2010). In summary, there were no statistically significant differences in concentrations among the three sampling sites for any of the analytes, with the exception of OXC. OXC concentrations were below the LOQ at both the primary and secondary treatment sampling sites.

In general, the measured concentrations presented here compare well to data obtained by Leclercq et al. in their survey of CBZ, OXC, and metabolite concentrations at three sewage treatment plants in France (Leclercq et al., 2009) and Miao and Metcalfe in their survey of a WWTP in Ontario, Canada (Miao et al., 2005). In the published papers by Leclercq and Miao, CBZ-DiOH concentrations ranged from 311 to 1415 ng/L, compared to the 347 – 392 ng/L we present in **Table 5**. Because CBZ-DiOH is a metabolite of both CBZ and OXC, it was anticipated that this major metabolite would be detected at higher concentrations than either of

the parent drugs or primary metabolites (Miao and Metcalfe, 2003). Additionally, Leclercq et al. determined that CBZ-epoxide concentrations at influent and effluent sampling sites was less than 30 ng/L (Leclercq et al., 2009), which compared well to the concentrations of 10.3 ng/L or less as we determined. Miao determined the CBZ-epoxide had a mean concentration of 19.1 ng/L in treated wastewater at the Canadian WWTP (Miao et al., 2005). Concentrations of CBZ-10-OH ranged from below the LOQ to 1065 ng/L in the samples from France (Leclercq et al., 2009), and 32.5 ng/L in Canada (Miao et al., 2005) compared to the 354 to 429 ng/L levels determined here. The CBZ data compared favorably as well, with concentrations from 112 to 416 ng/L in the previously mentioned French and Canadian samples (Leclercq et al., 2009; Miao et al., 2005) and 117 ng/L in samples at a Spanish sewage treatment plant (Rosal et al., 2010) versus the 129 – 181 ng/L data presented here (**Table 5**). Trenholm and colleagues measured CBZ by on-line SPE with LC/MS/MS detection and found CBZ concentrations ranging from approximately 200 to 400 ng/L in wastewater effluent and water recharge basin samples from Nevada (Trenholm et al., 2009). The data was not consistent, however, with regards to OXC. We determined that concentrations of this parent drug ranged from 13.7 ng/L to below the LOQ at the three sampling sites. In the sampled French sewage treatment plants, the levels of OXC ranged from 51 to 476 ng/L. Furthermore, removal rates at the three sampled sewage treatment plants in the French study had widely varying removal rates of these drugs and their metabolites (Leclercq et al., 2009).

Analyte Stability

One hundred µL of Level 1 calibration standard (that did not contain the IS) were added to 125 mL effluent matrix samples. A set of three samples from each of the sampling areas was

processed by clean-up, extraction, and analysis by LC/MS/MS immediately after adding and mixing the aliquot of Level 1 calibration standard. A second set of three samples from each study area was stored at 4 °C for one week prior being process as described above. The results of the holding time study are shown in **Table 6**.

Briefly, the concentrations of CBZ, OXC, and metabolites recovered were comparable to results from the method validation experiments previously described. Generally, all the matrix spiked samples from each study area showed no significant change in measured analyte concentrations when comparing samples processed at day zero of experiment to samples stored at 4 °C and processed after seven days. Concentration measurements of CBZ-10-OH for the effluent matrix spikes of the primary treatment station were lower than the other two effluent sampling locations for both the day 0 and 7 processed samples. The mean of the day 7 samples from the primary effluents were the lowest CBZ-10-OH concentrations measured and notably less than day 0 ($P = 0.0330$; **Table 6**).

In summary, a method for the quantitation of CBZ, OXC, and metabolites using an isotopically-labeled internal standard was validated and used to assess concentrations of this class of compounds at a U.S.-based wastewater treatment plant. Future work will focus on using this quantitative method to characterize CBZ, OXC, and metabolite concentrations in sampled groundwater to determine the extent of contamination by WWTP effluent. Additionally, future plans include the sampling of surface waters and San Francisco Bay water to determine the extent of further contamination and mixing downstream of our sampled WWTP.

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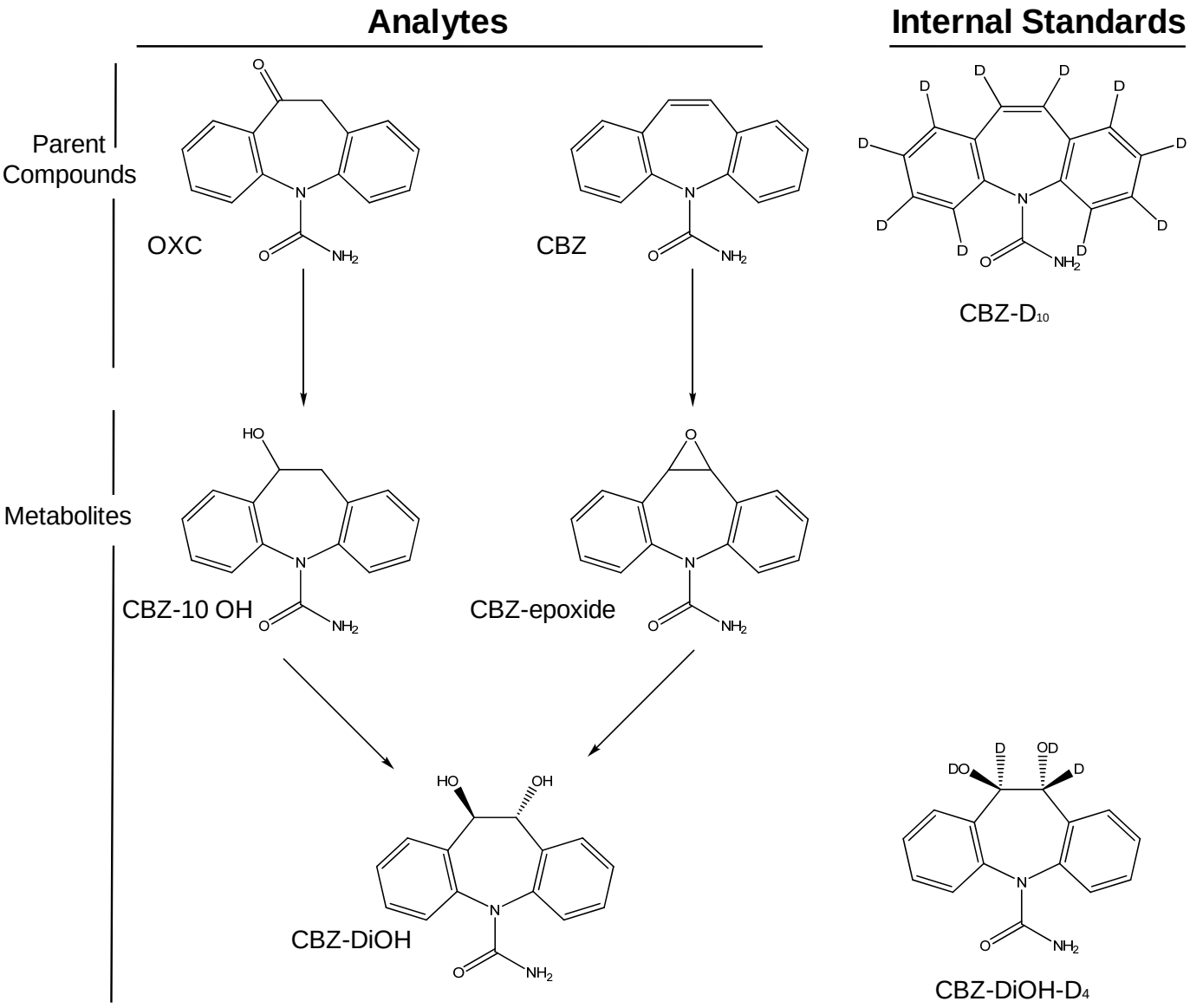

Figure 1: Analytes. Structures of analytes carbamazepine (CBZ) and oxcarbazepine (OXC) and their metabolites, including carbamazepine-epoxide (CBZ-epoxide), 10, 11-dihydro-10-hydroxycarbamazepine (CBZ-10OH), 10,11-dihydro-10,11-dihydroxycarbamazepine (CBZ-DiOH), and internal standards CBZ-D_{10} and CBZ-DiOH-D_4.

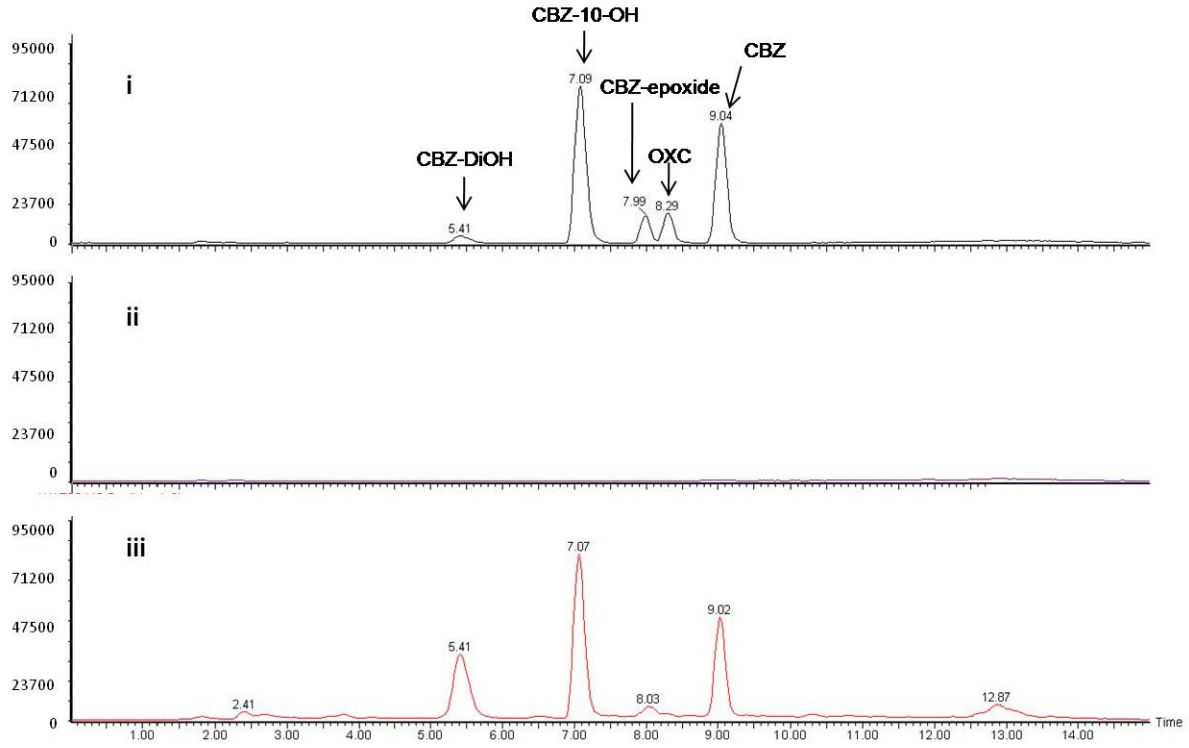

Figure 2: Chromatograms. Example chromatograms of count rate versus elution time of a standard at Level 6 (i), reagent water blank prepared by solid phase extraction (SPE) (ii), and composite water sample prepared by SPE as described (iii).

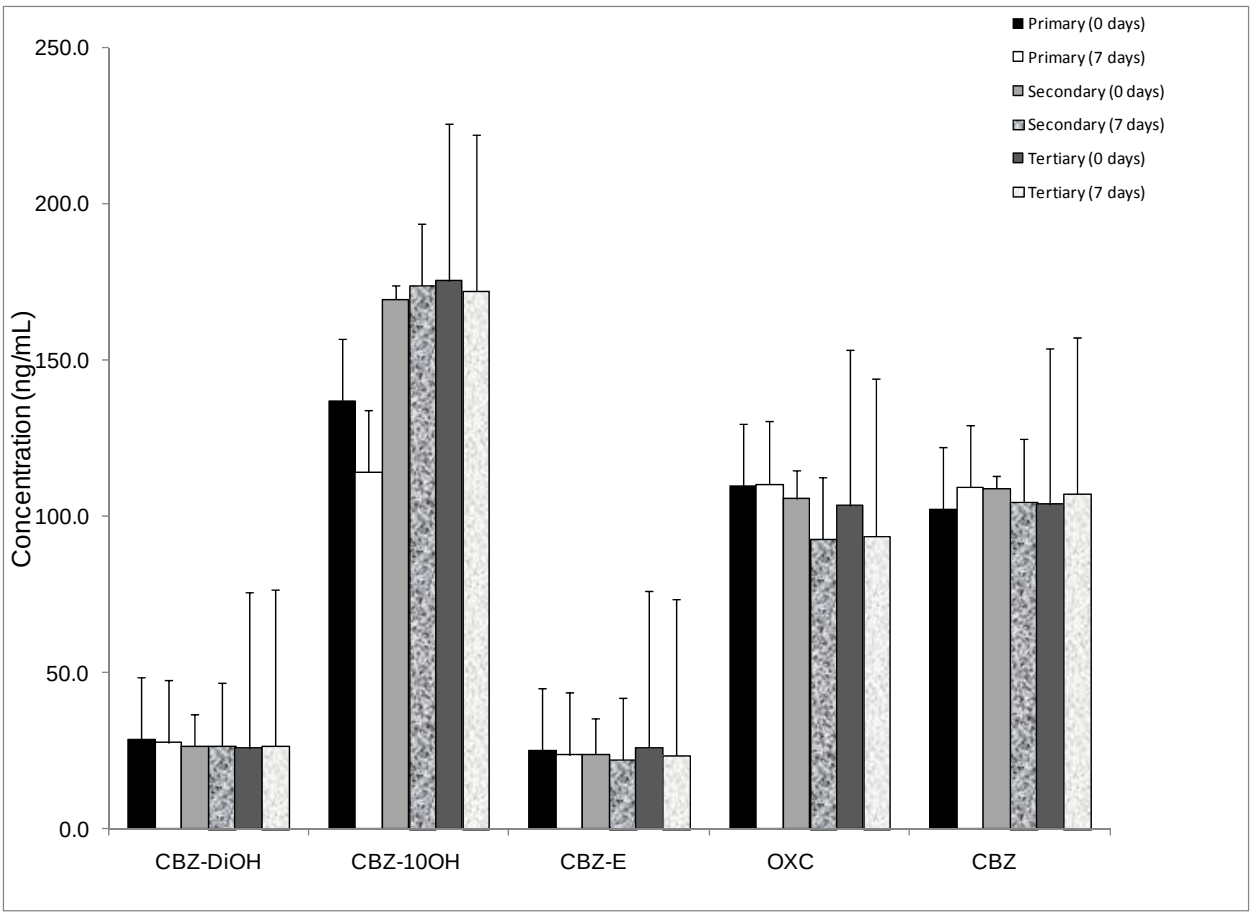

Figure 3: Analyte Stability. Holding time study of primary, secondary, and tertiary effluents with mean concentrations of CBZ, OXC, and metabolites. Mean concentration (ng/mL) is represented by solid bar; uncertainty (1 SD) is represented by error bar.

Table 1: **Calibration Standards.**
Concentrations (ng/mL) of Analytes and IS in Calibration Standards Prepared in HPLC-Grade Water

standard level	analyte					IS
	CBZ	OXC	CBZ-epoxide	CBZ-10-OH	CBZ-DiOH	$CBZ\text{-}D_{10}$
1	1100	2080	560	2780	360	10
2	550	1040	280	1390	180	10
3	225	520	140	695	90	10
4	112.5	260	70	347.5	45	10
5	55	104	28	139	18	10
6	27.5	52	14	69.5	9	10
7	11	20.8	5.6	27.8	3.6	10
8	5.5	10.4	2.8	13.9	1.8	10
9	2.2	4.2	1.1	5.6	0.72	10
10	1.1	2.1	0.56	2.8	0.36	10

Table 2: Instrument Settings.

Mass Spectrometer Settings for Analysis of CBZ, OXC, their Metabolites and an Isotopically-Labeled Internal Standard (indicated by *) with Positive Electrospray Ionization

	quantitation ion			confirmation ion		
Analyte or IS*	transition	cone (V)	collision (eV)	transition	cone (V)	collision (eV)
CBZ	*m/z* 237.1 → *m/z* 194.2	25	20	*m/z* 237.1 → *m/z* 192.2	25	20
OXC	*m/z* 253.1 → *m/z* 236.3	15	10	*m/z* 253.1 → *m/z* 210.5	15	15
CBZ-epoxide	*m/z* 253.1 → *m/z* 180.2	15	15	*m/z* 253.1 → *m/z* 236.3	15	12
CBZ-10-OH	*m/z* 255.1 → *m/z* 236.1	15	10	*m/z* 255.1 → *m/z* 194.1	15	15
CBZ-DiOH	*m/z* 271.1 → *m/z* 253.4	15	15	*m/z* 271.1 → *m/z* 236.1	15	15
CBZ-D_{10} *	*m/z* 247.2 → *m/z* 204.3	25	18	-	-	-

Table 3: Method Figures of Merit.
Method Characteristics of CBZ, OXC, and Metabolites
as Determined by LC/MS/MS

				Intra-day variability (%)		Inter-day variability (%)
analyte	R^2	LOD (ng/mL)	LOQ (ng/mL)	Standard ($n \geq 3$)	Water Sample ($n = 3$)	Standard ($n = 6$)
CBZ	0.9918	< 1.1	1.1	6.9	25.0	15.6
OXC	0.9952	< 2.1	2.1	3.1	3.3	9.7
CBZ-epoxide	0.9917	< 0.56	0.56	11.4	19.9	13.0
CBZ-10-OH	0.9959	< 2.8	5.56	19.4	11.8	15.4
CBZ-DiOH	0.9982	1.8	3.6	11.3	10.3	12.3

Table 4: Method Verification

Measured background ("Control"), Added Spike, and Control + Added Spike concentrations (ng/mL) in composite effluent samples. Uncertainties (%RSD) are based on replicate experiments (n=3).

Analyte	Control (RSD) (ng/mL)	Added Spike (ng/mL)	Measured Control + Spike (ng/mL)	Recovery (RSD) (%)
CBZ	29.3 (13%)	27.5	59.6	105% (25%)
OXC	< LOQ	52.0	22.9	44% (3%)
CBZ-epoxide	1.6 (8%)	14.0	10.5	67% (7%)
CBZ-10-OH	85.3 (3%)	69.5	140.6	91% (20%)
CBZ-DiOH	75.7 (7%)	9.0	83.7	99% (57%)

Table 5: Quantification of CBZ, OXC, and Metabolites in Wastewater

Mean Concentrations (RSD, %) of CBZ, OXC, and Metabolites in Sampled Waters (n = 3), ng/L

Note:	Primary After primary, before secondary treatment	Secondary Chlorinated secondary effluent	Tertiary Tertiary (post-UV) effluent	*P* values by ANOVA
CBZ-DiOH	392 (4.5%)	354 (10%)	347 (10%)	0.2268
CBZ-10-OH	354 (1.0%)	429 (12%)	399 (8.5%)	0.2331
CBZ-epoxide	8.3 (15%)	10.3 (20%)	7.1 (12%)	0.0867
OXC	Below LOQ	Below LOQ	13.7 (26%)	0.0118
CBZ	153 (11%)	181 (26%)	129 (21%)	0.2350

Table 6: Analyte Stability

Matrix Spiking Level (ng/mL) and Mean Concentrations (ng/mL) of Primary, Secondary, and Tertiary Effluents (with Relative Standard Deviation, %) of CBZ, OXC, and Metabolite Analytes Extractions Processed at Day 0 (n=3) and Day 7 (n=3). Values indicated in bold font and boxed are significantly different at $P < 0.05$ (by Student's *t*-test) for Day 0 versus Day 7 analyses.

Analyte	Spike	Mean Concentration (%RSD)					
		Primary		Secondary		Tertiary	
		Day 0	Day 7	Day 7	Day 0	Day 0	Day 7
CBZ	110	102.2 (14.5)	109.2 (3.2)	108.8 (4.1)	104.8 (6.2)	103.8 (1.1)	107 (7.0)
OXC	208	109.5 (16.0)	110.3 (4.6)	105.7 (9.1)	92.6 (4.1)	103.3 (14.7)	93.9 (13.0)
CBZ-epoxide	56	25.2 (2.5)	23.7 (7.7)	23.9 (11.6)	22.1 (6.4)	26.0 (8.1)	23.5 (16.7)
CBZ-10-OH	278	**136.8 (7.2)**	**114.0 (3.6)**	169.4 (4.5)	173.6 (2.9)	175.4 (6.3)	171.9 (9.3)
CBZ-DiOH	36	28.7 (20.7)	27.5 (10.3)	26.5 (10.3)	26.7 (11.4)	25.8 (21.9)	26.7 (13.1)

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Appendix A:
Protocol for the Extraction, Clean-Up, and Analysis of Carbamazepine and Related Metabolites from Collected Water Samples by Solid Phase Extraction with Analysis by LC/MS/MS

Objective: This method describes the extraction of carbamazepine and related metabolites from water samples prior to analysis by liquid chromatography tandem mass spectrometry (LC/MS/MS).

Chemicals and Materials:

1. HPLC-grade solvents: methanol, reagent water, acetonitrile, and ethyl acetate
2. MS-grade mobile phase modifiers: formic acid
3. Standards: 100 µg/mL carbamazepine-D_{10} (used as internal standard) prepared in methanol from Cambridge Isotope Laboratories; ClinCal calibrant mix of carbamazepine (CBZ), oxcarbazepine (OXC), and metabolites (CBZ-DiOH, CBZ-10OH, and CBZ-epoxide)
4. Materials: Oasis HLB 150 mg/6 cc solid phase extraction (SPE) cartridges (Waters Corp, part no. 186003365, lot no. 088A39040B), XBridge C18 analytical HPLC column (Waters Corp., 15 mm x 2.1 mm i.d., 3.5 µm particle size), Teflon tubing with weights, SPE column fittings, peristaltic pump, autosampler vials and lids, autosampler inserts

Protocol:

1. Pre-clean all Teflon tubing with methanol by pumping methanol through tubing using peristaltic pump. Rinse Teflon tubing with reagent grade water after methanol wash before using for the clean-up of collected water samples.
2. Attach Oasis HLB SPE cartridges to yellow peristaltic pump tubing using the clear connectors. Turn the pump on and operate at speed 600.
3. Condition Oasis HLB SPE cartridges with 2 column volumes (about 12 mL) methanol followed by 1 column volume of reagent water. Once the reagent water has almost completely passed through the SPE cartridges, turn the peristaltic pump off. Refill the cartridges with one more column volume of distilled water.
4. Prepare water samples by adding 10 µL of internal standard (IS) CBZ-D_{10} (prepared at 1 µg/mL in methanol: to make this standard, mix 50 µL of 100 µg/mL CBZ-D_{10} stock solution with 4.950 mL of methanol. Mix well). Mix the water samples well. Water sample volumes range from 125 mL to 500 mL, depending on what the sample is and the expected concentration of CBZ.
5. Drop the weighted end of the Teflon tubing into the water sample that now contains the IS. The Teflon tubing has been fitted with a white SPE connector. Fit this connector piece into the top of the Oasis HLB SPE cartridge that contains 1 column volume of reagent water.
6. Make sure that the various fittings and joints of tubing connections in the system are air-tight. Turn on the peristaltic pump and make sure than the water sample

containing IS starts to get pumped through the Teflon tubing into the SPE cartridge. This may take several minutes.

7. Once the samples are reliably being pumped into the SPE cartridge, you can check occasionally throughout the day. A 125-mL sample will take about 4 hours to filter, whereas a 1 L sample might take close to 36 hours. Just make sure that the system's fittings are tight and that everything is well connected.
8. Once the entire water sample has been filtered through the SPE cartridge, you can remove the white SPE connector that holds the Teflon tubing. Leave the SPE cartridge connected to the yellow peristaltic pump tubing. Add ~ 2 mL reagent water to wash the column. Once the water and moved through the SPE sorbent bed, you can remove the SPE column from the peristaltic pump.
9. Put the SPE column onto a SPE vacuum manifold. Apply light vacuum to dry the column out of any excess water. Place an autosampler vial under the column to collect the following methanol and ethyl acetate fractions: first, add 0.5 mL methanol and apply the vacuum (be sure to collect this fraction!!!) and then add 0.5 mL ethyl acetate (collect this fraction into the same autosampler vial that contains the methanol fraction!!!). Cap the autosampler vial and mix well.
10. When you're ready for LC/MS/MS analysis, take a 200 µL aliquot of the methanol/ethyl acetate fraction and place into a clean autosampler vial. Using high-purity nitrogen, slowly and carefully evaporate the sample (this can be just to dryness). Add 75 µL reagent water and mix well. Place this new aqueous mix into an autosampler insert and cap the vial. Store at 4 °C until analysis.

LC/MS/MS Analysis:

1. A Waters 2795 Alliance high performance liquid chromatograph with a Waters Micromass Quattro Micro *api* triple quadrupole mass spectrometer (LC/MS/MS) is used for the analyses of CBZ and metabolites.
2. Calibrate the instrument monthly using a sodium iodide/cesium iodide calibrant. This solution can be found in the refrigerator in the lab.
3. The instrument was previously tuned for carbamazepine analyses. Use the following methods to run the instrument (note: all are found at C:\MassLynx\Janel\CBZ.pro)
    a. Tune file: CBZ.ipr
    b. Inlet file: CBZ.wat
    c. MS method: CBZ.exp
    d. Processing method: CBZ.mdb
4. The tune parameters are as follows:
    a. Capillary voltage: 3.0 kV (positive ESI)
    b. Cone voltage: depends on analyte (ranges from 15 to 25 V)
    c. RF lens: 0.2 V
    d. Extractor: 2 V
    e. Source temp: 120 °C
    f. Desolvation temp: 300 °C
    g. Desolvation gas: 500 L/h

h. Cone gas: 45 L/h
i. Low and high mass, resolution 1: 14.5
j. Ion energy 1: 0.5
k. Entrance: -1 eV
l. Collision energy, eV: depends on analyte and product ion (ranges from 10 - 20 eV)
m. Exit energy: 2 eV
n. Low and high mass, resolution 2: 15.0
o. Ion energy 2: 1.5
p. Multiplier: 650
q. Inter-scan delay: 0.1
r. Span: 0
s. Dwell time: 0.1 ms
t. Inter-channel delay: 0.2
u. Repeats: 1

5. HPLC parameters:
   a. Column: Waters XBridge C18 (150 x 2.1 mm i.d., 3.5 µm)
   b. Mobile phase A: Water + 0.1% formic acid
   c. Mobile phase B: Acetonitrile + 0.1% formic acid
   d. Strong needle wash: 40/40/20 acetonitrile/methanol/water
   e. Weak needle wash: 90/10 water/acetonitrile
   f. Injection volume: 20 µL
   g. Flow rate: 200 µL/min
   h. Gradient: 0 – 2 min (80% A, 20% B) ramped to 20%A/80%B at 8 min (hold for 2 min), increase to 80%A/20%B at 11 min (hold for 4 min).
   i. Column equilibration time between runs: 2 min
   j. Column temperature: 30 °C (± 5 °C)
   k. Autosampler temperature: 4 °C (± 2 °C)

6. Summary of HPLC and MS characteristics:

Analyte	Parent ion (m/z)	Product Ions (m/z)	Cone (V)	Collision (eV)	RT (min)	MS Channel	Conc
CBZ	237.08	194.2	25	20	9.02	2	E
		192.2	25	20	9.02	1	
OXC	253.10	236.28	15	10	8.28	6	D
		210.45	15	15	8.28	5	
CBZ-epoxide	253.10	236.10	15	12	7.94	4	C
		180.20	15	15	7.97	5	
CBZ-10OH	255.07	236.97	15	10	7.04	8	B
		194.07	15	15	7.04	7	
CBZ-DiOH	271.10	236.09	15	15	5.35	9	A
		253.39	15	15	5.35	10	
CBZ-D10 (IS)	247.15	204.28	25	18	9.02	3	F