Anal. Chem. **2003,** *75,* 3731−3738

Determination of Carbamazepine and Its Metabolites in Aqueous Samples Using Liquid Chromatography−Electrospray Tandem Mass Spectrometry

Xiu-Sheng Miao and Chris D. Metcalfe*

Water Quality Centre, Trent University, Peterborough, Ontario K9J 7B8, Canada

A quantitative method is described for solid-phase extraction (SPE) followed by liquid chromatography−tandem mass spectrometry (LC−MS/MS) for the simultaneous analysis of carbamazepine and its five metabolites, 10,11-dihydro-10,11-epoxycarbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine, 2-hydroxycarbamazepine, 3-hydroxycarbamazepine, and 10,11-dihydro-10-hydroxycarbamazepine. An SPE procedure was used to concentrate target compounds from aqueous samples collected from sewage treatment plant (STP) wastewater and surface water. Extracts were analyzed using electrospray LC−MS/MS with time-scheduled selected reaction monitoring. The recoveries of the analytes were 83.6−102.2% from untreated sewage (influent), 90.6−103.5% from treated sewage (effluent), and 95.7−102.9% from surface water samples. The instrumental detection limits were 0.8−4.8 pg for the analytes. Matrix effects were investigated for the analytes in HPLC-grade water, surface water, and STP influent and effluent. Ion suppression increased for analytes in order of surface water to STP effluent to STP influent, but no ion suppression was observed for analytes in HPLC-grade water. The developed method was validated by analysis of environmental aqueous samples: STP influent and effluent and surface water. Carbamazepine and all five metabolites were detected in STP influent and effluent samples. Only carbamazepine and 10,11-dihydro-10,11-dihydroxycarbamazepine were detected in the surface water sample. Notably, 10,11-dihydro-10,11-dihydroxycarbamazepine was detected at ∼3 times higher concentrations than the parent drug, carbamazepine, in all of the aqueous samples. To our knowledge, this is the first report on the simultaneous determination of carbamazepine and its metabolites in environmental samples.

Pharmaceutically active compounds, including drugs and their active metabolites, are an emerging environmental issue, due to their presence in the aquatic environment and possible impact on wildlife and on humans.[1−3] Carbamazepine (proprietary name Tegretol) (5*H*-dibenzo[*b*,*f*]azepine-5-carboxamide) is an important drug for the treatment of epilepsy, which is the second most common central neuron system disease after stroke. Its efficacy, together with its acceptable safety profile, has made carbamazepine the first-choice antiepileptic drug for a wide range of seizure disorders in adults and children. Carbamazepine has been available widely as an anticonvulsant for more than thirty years[4] and has replaced both phenytoin and phenobarbitone as the first-choice anticonvulsant for a number of pediatric seizure disorders. In addition, carbamazepine is used for the treatment of trigeminal neuralgia and as a psychotropic agent.[5−7] Recently, carbamazepine has been introduced in clinical psychiatry for the treatment of schizophrenia because of its mood-stabilizing properties.[8,9] It is also used for treatment of bipolar disorder, which is a serious disease afflicting 1.2% of adults in the United Sates.[10,11] Finally, carbamazepine can be combined with other drugs for the treatment of alcohol withdrawal.[12]

Carbamazepine undergoes extensive hepatic metabolism by the cytochrome P450 (CYP) system.[13,14] Thirty-three metabolites of carbamazepine have been identified from human and rat urine.[15] The main metabolic pathway of carbamazepine is oxidation to 10,11-dihydro-10,11-epoxycarbamazepine, then hydration to 10,11-dihydro-10,11-dihydroxycarbamazepine, and conjugation of

* Corresponding author. Phone (705) 748-1011, ext 1272. Fax: (705) 748-1587' E-mail: cmetcalfe@trentu.ca.

(1) Daughton, C. G.; Ternes, T. A. *Environ. Health Perspect.* **1999**, 107 (Suppl. 6), 907−938.
(2) Golet, E. M.; Alder, A. C.; Hartmann, A.; Ternes, T. A.; Giger, W. *Anal. Chem.* **2001**, *73*, 3632−3638.
(3) Kolpin, D. W.; Furlong, E. T.; Meyer, M. T.; Thurman, E. M.; Zaugg, S. D.; Barber, L. B.; Buxton, H. T. *Environ. Sci. Technol.* **2002**; *36*, 1202−1211.
(4) Bernus, I.; Dickinson, R. G.; Hooper, W. D.; Eadie, M. J. *Epilepsy Res.* **1996**, *24*, 163−172.
(5) Deleu, D.; Aarons, L.; Ahmed, I. A. *Eur. J. Clin. Pharmacol.* **2001**, *57*, 243−248.
(6) Albani, F.; Riva, R.; Baruzzi, A. *Pharmacopsychiatry* **1995**, *28*, 235−244.
(7) Duche, P.; Loiseau, B. In *Antiepileptic Drugs*, 4th ed.; Levy, R. H., Mattson, R. H., Meldrum, B. S., Penry, J. K., Dreyfuss, F. E., Eds.; Raven Press: New York, 1995; p 555.
(8) Okuma, T. *Neuropsychobiology* **1993**, *27*, 138−145.
(9) Emrich, H. M.; Dose, M. Wolf, R. *Neuropsychobiology* **1993**, *27*, 158−165.
(10) Dilsaver, S. C.; Swann, S. C.; Chen, Y.-W.; Shoaib, A.; Joe, B.; Krajewski, K. J.; Gruber, N.; Tsai, Y. *Biol. Psychiatry* **1996**, *40*, 935−937.
(11) Weissman, M. M.; Leaf, P. J.; Tischler, G. L. *Psychol. Med.* **1988**, *44*, 141−153.
(12) Franz, M.; Dlabal, H.; Kunz, S.; Ulferts, J.; Gruppe, H.; Gallhofer, B. *Eur. Arch. Psychiatry Clin. Neurosci.* **2001**, *251*, 185−192.
(13) Kerr, M.; Thummel, K. E.; Wurden, C. J.; Klein, S. M.; Kroetz, D. L.; Gonzalez, F. J.; Levy, R. H. *Biochem. Pharmacol.* **1994**, *47*, 1969−1979.
(14) Valentine, C. R.; Valentine, J. L.; Seng, J.; Leakey, J.; Casciano, D. *Cell Biol. Toxicol.* **1996**, *12*, 155−165.
(15) Lertratanangkoon, K.; Horning, M. *Drug Metab. Dispos.* **1982**, *10*, 1−10.

10.1021/ac030082k CCC: $25.00
Published on Web 07/02/2003

10,11-dihydro-10,11-dihydroxycarbamazepine with glucuronide. The hydrolysis of 10,11-dihydro-10,11-epoxycarbamazepine to 10,11-dihydro-10,11-dihydroxycarbamazepine is catalyzed by microsomal epoxide hydrolase.[16] The metabolism of carbamazepine to 10,11-dihydro-10,11-epoxycarbamazepine appears to be catalyzed by CYP3A4 and CYP2C8.[13] Lesser pathways include the oxidation to 2-hydroxycarbamazepine and 3-hydroxycarbamazepine,[17] which appear to be catalyzed by CYP1A2, and oxidation to 10,11-dihydro-10-hydroxycarbamazepine.

The most important metabolites are 10,11-dihydro-10,11-dihydroxycarbamazepine and, to a lesser extent, 10,11-dihydro-10,11-epoxycarbamazepine. The latter compound has been shown to possess similar antiepileptic properties to carbamazepine and it may cause neurotoxic effects.[18,19] In some cases, clinical toxicities parallel 10,11-dihydro-10,11-epoxycarbamazepine concentration.[20] Despite being chemically stable under physiological conditions, 10,11-dihydro-10,11-epoxycarbamazepine is converted to the 10,11-dihydro-10,11-dihydroxycarbamazepine metabolite by epoxide hydrolase. Bernus et al.[21] investigated the metabolism of carbamazepine in women during pregnancy, and found that carbamazepine, 10,11-dihydro-10,11-epoxycarbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine, carbamazepine-acridan, 2-hydroxycarbamazepine, and 3-hydroxycarbamazepine accounted for 0.5, 2.1, 34.6, 3.2, 2.3, and 3.7% of total concentrations in urine samples, respectively. The 10,11-dihydro-10,11-dihydroxycarbamazepine metabolite is not pharmaceutically active.[7]

Environmental field studies have shown that carbamazepine is one of the most frequently detected pharmaceuticals in sewage treatment plant (STP) effluent, in river water,[22] and in seawater.[23] Carbamazepine has been used to evaluate the efficiency of removal of pharmaceuticals in STPs.[24] However, there are no data on the fate of the metabolites of carbamazepine in the environment. Because of the high proportion of carbamazepine metabolites in biological fluids, there is reason to suspect that the metabolites will be present in domestic sewage and in the aquatic environment near STP discharges. Therefore, sensitive and specific analytical methods are required to detect these analytes at trace levels (ppb or lower). Analytical methods for therapeutic drug monitoring in biological fluids were established for clinical purposes[25−27] and are not suitable for environmental monitoring. Our aim is to establish a sensitive, specific, and reproducible analytical technique for the simultaneous determination of carbamazepine and five of its major metabolites (Table 1) in aqueous environmental matrixes using solid-phase extraction (SPE), followed by analysis with liquid chromatography−electrospray tandem mass spectrometry (LC−ES-MS/MS).

EXPERIMENTAL SECTION

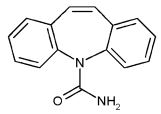
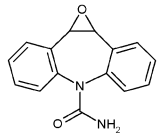
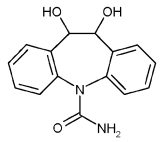
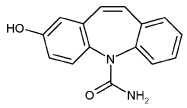
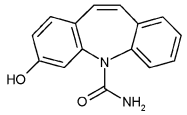
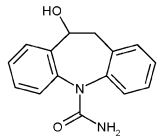
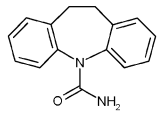
Chemicals and Standards. Carbamazepine, 10,11-dihydro-10,11-epoxycarbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine, 2-hydroxycarbamazepine, 3-hydroxycarbamazepine, and 10,11-dihydro-10-hydroxycarbamazepine were provided by Novartis Pharma AG (Basel, Switzerland). Dihydrocarbamazepine was provided by Dr. Thomas Ternes (ESWE-Institute for Water Research and Water Technology, Wiesbaden, Germany). Acetonitrile and methanol were purchased from Caledon Laboratories (Georgetown, ON, Canada). Formic acid (90%) and HPLC-grade water were purchased from EM Science Industries (Gibbstown, NJ). Ammonium acetate (98%) was supplied by Sigma (St. Louis, MO).

Tandem Mass Spectrometry. Mass spectrometry was performed using a Quattro LC tandem quadrupole mass spectrometer (Micromass, Manchester, U.K.) equipped with a Z-Spray electrospray ionization source. The capillary was held at 3.5 kV, and the cone was operated at an optimized voltage for each analyte in positive-ion mode. Nitrogen was used as the drying and nebulizing gas at flow rates of 450 and 70 L/h, respectively. The source and desolvation temperatures were optimized under LC−MS/MS conditions, which were 100 and 350 °C, respectively. MassLynx v 3.4 software was applied for data acquisition and processing. During the method development, individual standard solutions were infused through a syringe pump (Harvard Apparatus, Holliston, MA) at a flow rate of 10 μL/min into the mass analyzer. Following the selection of precursor ions, $[M + H]^+$, by the first quadrupole mass analyzer, collision-induced dissociation (CID) was carried out using 1.0×10^{-3} mbar UHP argon (Praxair, Peterborough, ON, Canada) in the hexapole collision cell in the range 0−30 eV. Product ion mass spectra were obtained at a series of collision energies to determine the optimal collision energy for each analyte based on the relative intensities of the selected product ions. The mass spectrometer was operated in selected reaction monitoring (SRM) mode with unit resolution on both the first and second quadrupole analyzers. A dwell time of 200 ms per ion pair was used, and the interchannel delay was 0.01 s.

Liquid Chromatography. Analyte separations were conducted with an Alliance 2695 liquid chromatograph (Waters, Milford, MA) with a Genesis C_8 column (150 × 2.1 mm i.d., 3 μm; Jones Chromatography, Hengoed, Mid Glamorgan, U.K.). The mobile phases were as follows: mobile phase A, acetonitrile/methanol (2:3, v/v); mobile phase B, 10 mM ammonium acetate with 0.1% formic acid (pH 4.0). The flow rate was 0.2 mL/min, and the mobile phases were degassed with an in-line degasser. The mobile-phase gradient used was held at 45% A for 6 min, then increased linearly to 100% within 1 min and held for 2 min at 100% of A, and then ramped back to 45% of A in 2 min. The injection volume was 20 μL, and the retention times were typically 3.6−10.4 min for all six analytes and the internal standard, dihydrocarbamazepine.

(16) Kitteringham, N. R.; Davis, C.; Howard, N.; Pirmohamed, M.; Park, B. K. *J. Pharmacol. Exp. Ther.* **1996**, *278*, 1018−1027.
(17) Myllynen, P.; Pienimäki, P.; Raunio, H.; Vähäkangas, K. *Hum. Exp. Toxicol.* **1998**, *12*, 668−676.
(18) Semah, F.; Gimnez, F.; Longer, E.; Laplane, D.; Thuillier, A.; Baulac, M. *Ther. Drug Monit.* **1994**, *16*, 537−540.
(19) Rambeck, B.; Schnabel, R.; May, T.; Jürgens, U.; Villagran, R. *Ther. Drug Monit.* **1993**, *15*, 91−98.
(20) Mesdjian, E.; Sérée, E.; Charvet, B.; Mirrione, A.; Bourgarel-Rey, V.; Desobry, A.; Barra, Y. *Life Sci.* **1999**, *64*, 827−835.
(21) Bernus, I.; Hooper, W. D.; Dickinson, R. G.; Eadie, M. J. *Epilepsy Res.* **1995**, *21*, 65−75.
(22) Metcalfe, C. D.; Miao, X.-S.; Koenig, B. G.; Struger, J. *Environ. Toxicol. Chem.* **2003** (in press).
(23) Weigel, S.; Bester, K.; Hühnerfuss, H. *J. Chromatogr., A* **2001**, *912*, 151−161.
(24) Ternes, T. A.; Meisenheimer, M.; McDowell, D.; Sacher, F.; Brauch, H.-J.; Haist-Gulde, B.; Preuss, G.; Wilme, U.; Zulei-Seibert, N. *Environ. Sci. Technol.* **2002**, *36*, 3855−3863.
(25) Mandrioli, R.; Albani, F.; Casamenti, G.; Sabbioni, C. *J. Chromatogr., B* **2001**, *762*, 109−116.
(26) Raggi, M. A.; Pucci, V.; Maurizio, A.; Muzikar, J.; Kenndler, E. *J. Chromatogr., B* **2002**, 217−225.
(27) van Rooyen, G. F.; Badenhorst, D.; Swart, K. J.; Hundt, H. K. L.; Scanes, T.; Hundt, A. F. *J. Chromatogr., B* **2002**, *769*, 1−7.

Table 1. Molecular Structures of Carbamazepine, Its Metabolites, and the Internal Standard

structure	analyte	abbreviation	formula and MW[a]
	carbamazepine	CBZ	$C_{15}H_{12}N_2O$ 236.10[a]
	10,11-dihydro-10,11-epoxycarbamazepine	CBZ-EP	$C_{15}H_{12}N_2O_2$ 252.09
	10,11-dihydro-10,11-dihydroxycarbamazepine	CBZ-DiOH	$C_{15}H_{14}N_2O_3$ 270.10
	2-hydroxycarbamazepine	CBZ-2OH	$C_{15}H_{12}N_2O_2$ 252.09
	3-hydroxycarbamazepine	CBZ-3OH	$C_{15}H_{12}N_2O_2$ 252.09
	10,11-dihydro-10-hydroxycarbamazepine	CBZ-10OH	$C_{15}H_{14}N_2O_2$ 254.10
	10,11-dihydrocarbamazepine[b]	CBZ-DiH	$C_{15}H_{14}N_2O$ 238.11

[a] Molecular weight (MW) was calculated with the lowest isotopomer. [b] Surrogate internal standard

Sample Collection. Samples of untreated sewage (influent) and treated final sewage (effluent) were collected from an STP in Peterborough, ON, Canada, on November 6, 2002. The STP currently serves a population of about 75 000 people. Its design average flow capacity is 60 000 m^3/day, and its average handling flow is presently 46 000 m^3/day (77% of its design capacity). The influent flow rate consists of about 25% industrial wastewater and 75% domestic sewage. The treatment process includes grit removal and screening, primary treatment, and secondary treatment, followed by seasonal chlorine disinfection. The STP releases its treated final effluent to the Otonabee River. Surface water was collected from the Otonabee River at a sampling site ~100 m below the outlet of the STP. The samples were collected in solvent-washed amber glass bottles, stored in a cold room at 4 °C, and extracted within 24 h.

Sample Extraction. To remove suspended material, the aqueous samples were vacuum filtered through 1.5-μm glass microfiber filters, which had been prewashed with hexane/dichloromethane (1:1) in a Soxhlet apparatus. After filtration, the pH of samples was adjusted to 7.0 with 3.0 M H_2SO_4, 1.0 M NaOH, or both.

Analytes were extracted using 6 cm^3/500 mg Oasis hydrophilic−lipophilic balance (HLB) SPE cartridges from Waters, because they are designed to retain both hydrophilic and hydrophobic compounds with high capacity. The SPE cartridges were installed on a vacuum manifold and preconditioned sequentially with 6 mL of acetone, 6 mL of methanol, and 6 mL of HPLC-grade water (pH 7.0). Thereafter, the aqueous samples were allowed to pass slowly through the cartridges at a rate of ~10 mL/min. After passage of the samples, the sample bottles were rinsed with 10 mL of HPLC-grade water (pH 7.0), and the rinses were allowed to flow through the cartridges. The cartridge was dried under vacuum for 1 min and was eluted with three 2-mL portions of methanol. Each aliquot of methanol was eluted through

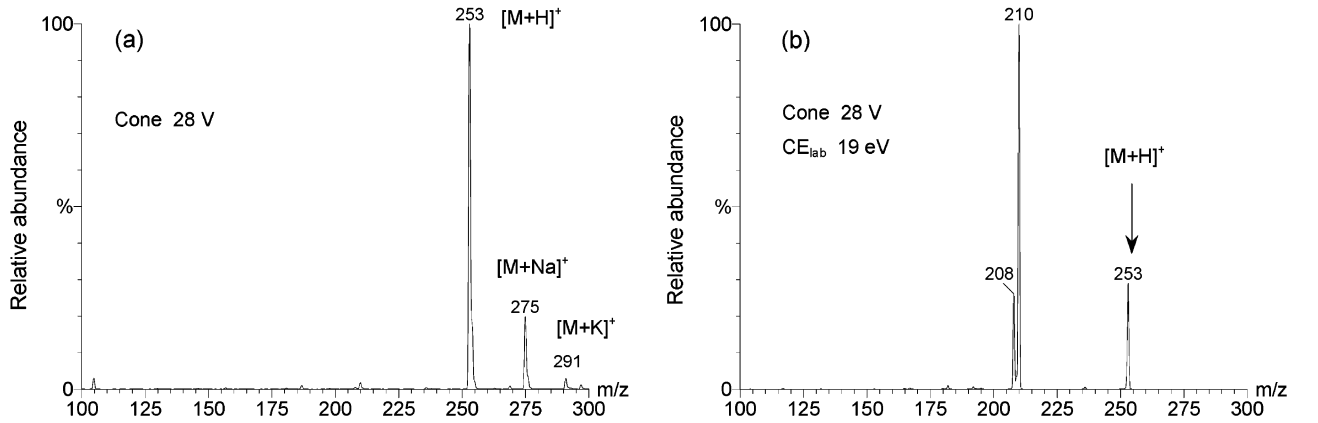


Figure 1. (a) Full-scan mass spectrum of 2-hydroxycarbamazepine and (b) product ion mass spectrum of $[M + H]^+$ for 2-hydroxycarbamazepine. The selected precursor ion is indicated with a vertical arrow.

the column for a minimum of 10 min. The eluates were collected in a 10-mL test tube and concentrated to almost dryness with a UVS 400 vacuum centrifuge (Savant Instruments). The samples were reconstituted to 0.25 mL with methanol/water (3:2). Care was taken to not allow the samples to go to dryness.

Method Validation. Recovery experiments with spiked samples were performed to determine the precision and accuracy of the method. Because detectable concentrations of analytes may be found in the aqueous samples, standard addition experiments were performed to determine the recoveries of analytes. The percent recovery of an analyte spiked into each type of aqueous sample was calculated as the measured spiked concentration minus the original sample concentration divided by the concentration added to the sample, multiplied by 100. The instrumental detection limit was defined as the lowest concentration of an analyte that yielded an ion signal with a signal-to-noise ratio of 3:1.

HPLC-grade water, surface water, and STP effluent and influent were used as sample matrixes to investigate matrix effects. To avoid losses due to extraction, the extracts from each of the matrixes were spiked with the standard solution after the extraction procedures and then analyzed using LC−ES-MS/MS. The signal suppression was calculated using the fractional recovery obtained from matrix samples spiked with the analyte standard. The fractional recovery (relative signal response) was calculated by relating the peak area obtained from a standard versus the peak area of the standard in a matrix sample. A value of 1.0 is indicative of no signal suppression or enhancement.

Data Analysis and Quantification. Peak areas, regression parameters, and concentrations were obtained by using the quantification portion of MassLynx software. Aliquots of 1.0-mL standard solutions of analytes at five different concentrations containing the surrogate internal standard, dihydrocarbamazepine, were added to 0.25 L of influent, 0.5 L of effluent, and 1.0 L of surface water samples. A constant concentration of 100 ng/mL internal standard was added to all samples. The analyte peak areas were generated from the spiked samples, with corrections for background concentrations of the analytes in the samples. The peak areas were plotted against the corresponding concentrations of the analytes, and calibration curves were calculated by the least-squares method. Response factors of the analytes to internal standard in different matrixes were calculated from the calibration curves and were used to quantify the analytes in each type of aqueous sample. Fluctuations in the signal intensity were monitored by analyzing a standard solution at the start and the end of each set of analyses.

Table 2. Optimized LC−ES-MS/MS Conditions for the Analysis of Carbamazepine and Its Metabolites

analyte	time window (min)	SRM (m/z)	cone voltage (V)	collision energy (eV)	instrument detection limit (pg)
CBZ	8.6−10.5	237 → 194	28	19	0.8
CBZ-EP	4.8−5.8	253 → 180	24	24	2.0
CBZ-DiOH	3.2−4.2	271 → 253	22	8	4.8
CBZ-2OH	4.2−5.2	253 → 210	28	20	1.0
CBZ-3OH	5.4−6.4	253 → 210	28	20	1.2
CBZ-10OH	3.8−5.0	255 → 237	20	10	2.1
CBZ-DiH[a]	9.6−11.6	239 → 194	28	23	

[a] Internal standard.

RESULTS AND DISCUSSION

ES-MS/MS. ES-MS/MS was performed in positive-ion mode using direct infusion of individual standard solutions of the analytes. Figure 1a shows a full-scan mass spectrum of 2-hydroxycarbamazepine, where the ion signal for $[M + H]^+$ (m/z 253) is present at the greatest intensity, compared to $[M + Na]^+$ and $[M + K]^+$ at m/z 275 and 291, respectively. Following the optimization of the capillary voltage, the sample cone voltage was optimized for each analyte based on its ion signal intensity of the protonated molecule (Table 2).

Following the selection of the precursor ion and optimization of the ion signal intensity, CID was carried out by adjusting the collision energy in the hexapole collision cell at a fixed collision gas pressure (1.0×1.0^{-3} mbar). Figure 1b shows the product ion mass spectrum of the protonated 2-hydroxycarbamazepine. The only major product ion was observed at m/z 210, corresponding to loss of the structurally characteristic carbamoyl group (HNCO, 43 Da). CID of 3-hydroxycarbamazepine generated a product ion mass spectrum almost identical with that of isomeric 2-hydroxycarbamazepine. The only major ion m/z 194 corresponds to a neutral loss of HNCO from $[M + H]^+$, which was observed in the product ion mass spectrum of protonated carbamazepine. Therefore, SRM channels were set at m/z 237 → 194 for carbamazepine and m/z 253 → 210 for 2-hydroxycarbamazepine and 3-hydroxycarbamazepine.

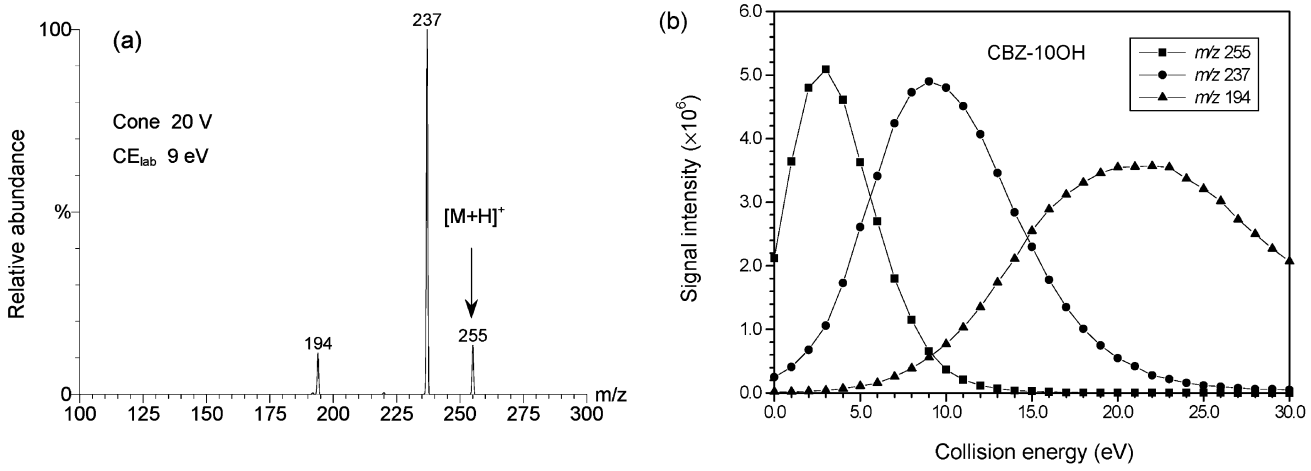


Figure 2. (a) Product ion mass spectrum of protonated 10,11-dihydro-10-hydroxycarbamazepine and (b) ion dissociation and formation as a function of collision energy in the laboratory frame for protonated 10,11-dihydro-10-hydroxycarbamazepine. The selected precursor ion is indicated with a vertical arrow.

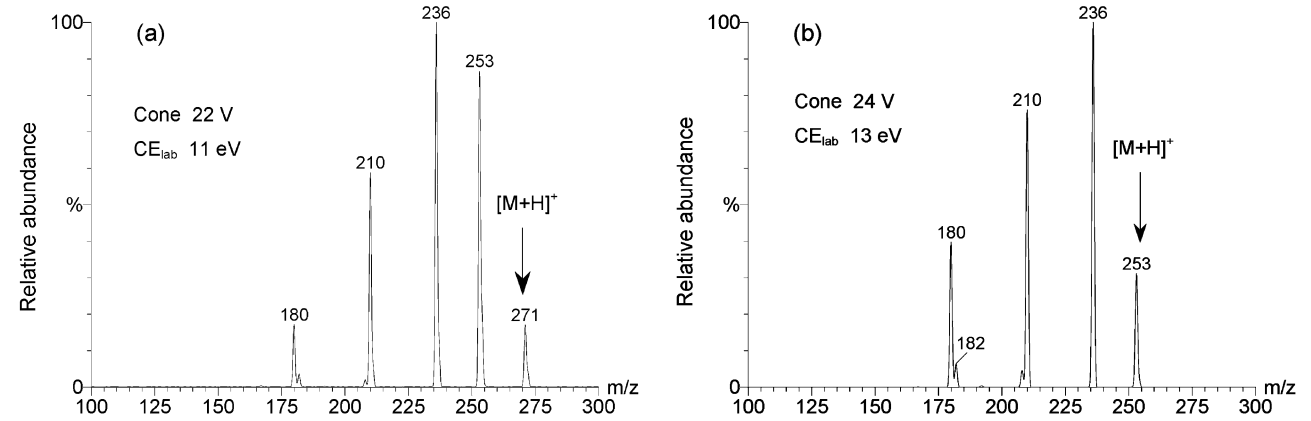


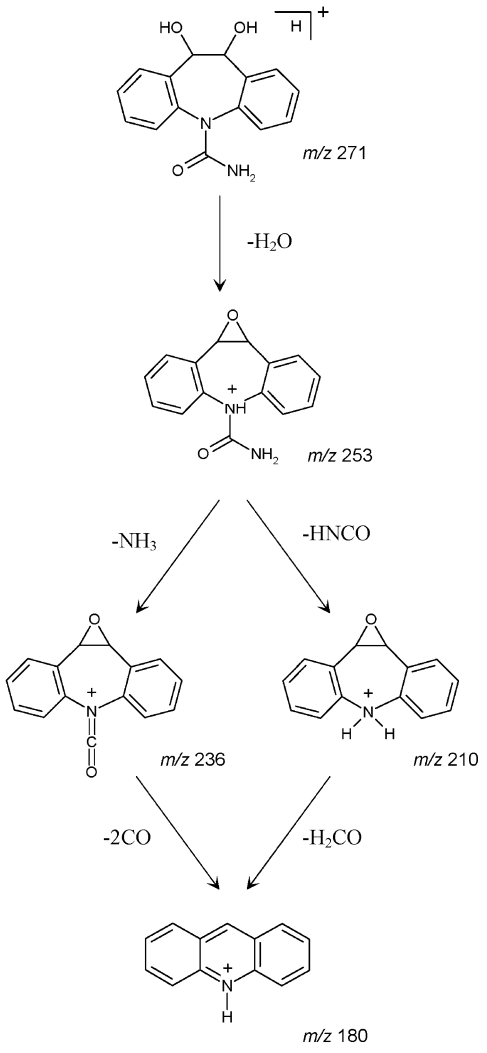
Figure 3. Product ion mass spectra of protonated (a) 10,11-dihydro-10,11-dihydroxycarbamazepine and (b) 10,11-dihydro-10,11-epoxycarbamazepine. The selected precursor ions are indicated with vertical arrows.

Compared to the simple product ion mass spectra observed in the CID of carbamazepine, 2-hydroxycarbamazepine, and 3-hydroxycarbamazepine, relatively complex product ion mass spectra were observed for the protonated molecules of 10,11-dihydro-10,11-epoxycarbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine, and 10,11-dihydro-10-hydroxycarbamazepine. Figure 2a shows the product ion mass spectrum of protonated 10,11-dihydro-10-hydroxycarbamazepine, consisting of two major ions at m/z 237 and 194, which correspond to losses of H_2O (18 Da) and HNCO and H_2O, respectively. The product ion profiles as a function of collision energy are shown in Figure 2b. In Figure 2b, the highest ion signal intensity of the parent ion, m/z 255, was observed at a collision energy of 3 eV in the laboratory frame with focusing effect; the highest intensities of the product ions m/z 237 and 194 were observed at collision energies of 10 and 21 eV, respectively. The product ion signal intensities under optimal conditions with SRM channel m/z 255 → 237 provide better sensitivity than m/z 255 → 194.

More product ion species were generated with CID of protonated 10,11-dihydro-10,11-dihydroxycarbamazepine and 10,11-dihydro-10,11-epoxycarbamazepine compared with CID of the other analytes. Figure 3 shows the product ion mass spectra of (a) 10,11-dihydro-10,11-dihydroxycarbamazepine and (b) 10,11-dihydro-10,11-epoxycarbamazepine. The CID pathways of 10,11-dihydro-10,11-epoxycarbamazepine are similar to those of 10,11-dihydro-10,11-dihydroxycarbamazepine, except for H_2O loss from the protonated molecule of 10,11-dihydro-10,11-dihydroxycarbamazepine. Scheme 1 illustrates the proposed fragmentation pathways of protonated 10,11-dihydro-10,11-dihydroxycarbamazepine. By loss of H_2O, the protonated molecule of 10,11-dihydro-10,11-dihydroxycarbamazepine generated ion m/z 253, which further yielded ions at m/z 210 and 236 by losses of HNCO (43 Da) and NH_3 (17 Da), respectively. Ions of m/z 210 and 236 could both yield the product ion of m/z 180 by rearrangement of the six-membered ring and losses of H_2CO (30 Da) and 2CO (56 Da), respectively. Based on the results from LC−MS/MS, SRM channels, m/z 271 → 253 and m/z 253 → 180 were selected for monitoring 10,11-dihydro-10,11-dihydroxycarbamazepine and 10,-11-dihydro-10,11-epoxycarbamazepine, respectively.

Chromatographic Separation. Due to the similar structures and CID processes of carbamazepine and its metabolites, there was potential for cross-talk among some SRM channels. In particular, the same SRM channel was used for analysis of 2-hydroxycarbamazepine and 3-hydroxycarbamazepine. In addition, 10,11-dihydro-10,11-dihydroxycarbamazepine can be observed in channels m/z 253 → 210 and m/z 253 → 180 that are used for monitoring 2-hydroxycarbamazepine and 3-hydroxycarbamazepine, and for 10,11-dihydro-10,11-epoxycarbamazepine, respectively.

Scheme 1. Proposed Fragmentation Pathways for 10,11-Dihydro-10,11-dihydroxycarbamazepine



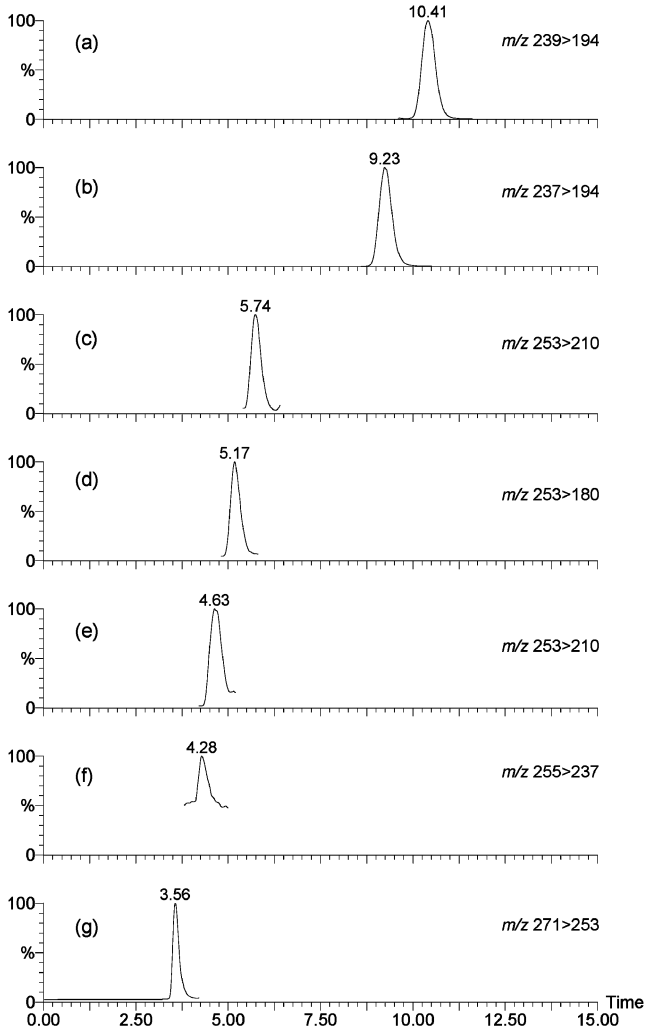


Figure 4. Time-scheduled SRM chromatograms of carbamazepine and its metabolites in an effluent sample from the STP of Peterborough, ON: (a) CBZ-DiH (internal standard), (b) CBZ, (c) CBZ-3OH, (d) CBZ-EP, (e) CBZ-2OH, (f) CBZ-10OH, and (g) CBZ-DiOH.

Therefore, chromatographic separation of analytes was critical for the determination of these compounds. Genesis C_{18} (150 × 2.1 mm i.d., 3 μm; Jones Chromatography) and polymer PLRP−S (150 × 2.1 mm i.d., 5 μm; Polymer Laboratories) columns were investigated as stationary phases for the separation of the analytes. However, a C_8 column (specifications as in the Experimental Section) showed the best chromatographic separation characteristics.

Initially, acetonitrile was used as the organic mobile-phase solvent for chromatographic separation, but 3-hydroxycarbamazepine and 10,11-dihydro-10,11-epoxycarbamazepine could not be completely resolved. 10,11-Dihydro-10,11-epoxycarbamazepine (SRM channel m/z 253 → 180) contributed to the SRM channel of m/z 253 → 210, which was used for monitoring 2-hydroxycarbamazepine and 3-hydroxycarbamazepine. A mixed organic solvent, acetonitrile/methanol (2:3), was used finally to improve the resolution of the above two compounds. The time-scheduled SRM chromatogram of the analytes is shown in Figure 4 for an STP effluent sample from Peterborough, ON. Under the described conditions, all analytes and the internal standard were resolved chromatographically with a retention time of 11 min.

Extraction Efficiency and Matrix Effects. To extract the analytes from aqueous samples, SPE was investigated using three types of cartridges, HLB (Waters), Supelclean-18 (Supelco), and LC-18 (Supelco). The HLB cartridge was finally chosen for SPE because of its superior extraction efficiencies for all of the analytes. The overall recoveries of carbamazepine, its metabolites, and the internal standard were 95.7−102.9% in surface water, 90.6−103.5% in STP effluent, and 83.6−102.2% in STP influent (Table 3).

LC−ES-MS (or MS/MS) is often applied to the analysis of analyte mixtures in complex matrixes. However, one drawback associated with this technique is that it is susceptible to matrix-related signal suppression or enhancement, which are believed to result from the competition of the analyte ions and matrix components for access to the droplet surface for gas-phase emission.[28] The presence of coextracted matrix components may severely affect analyte quantitation by LC−ES-MS/MS.

HPLC-grade water, surface water, and STP effluent and influent were chosen to investigate matrix effects. Figure 5 clearly shows the effect of the different matrixes on the ion current of the

(28) Pascoe, R.; Foley, J. P.; Gusev, A. I. *Anal. Chem.* **2001**, *73*, 6014−6023.

Table 3. Recoveries and Standard Deviations of Carbamazepine and Its Metabolites from Surface Water and STP Influent and Effluent[a]

analyte	surface water, %	effluent, %	influent, %
CBZ	100.6 ± 2.6	90.6 ± 5.4	89.5 ± 4.6
CBZ-EP	102.9 ± 4.1	96.5 ± 3.2	90.1 ± 5.9
CBZ-DiOH	95.7 ± 4.8	97.1 ± 2.6	83.6 ± 4.9
CBZ-2OH	97.3 ± 5.5	94.5 ± 5.6	95.3 ± 2.4
CBZ-3OH	98.1 ± 5.3	102.9 ± 4.5	102.2 ± 3.1
CBZ-10OH	100.5 ± 2.6	103.5 ± 5.8	99.4 ± 5.8

[a] Recoveries are the average of six determinations at a concentration of 100 ng/L.

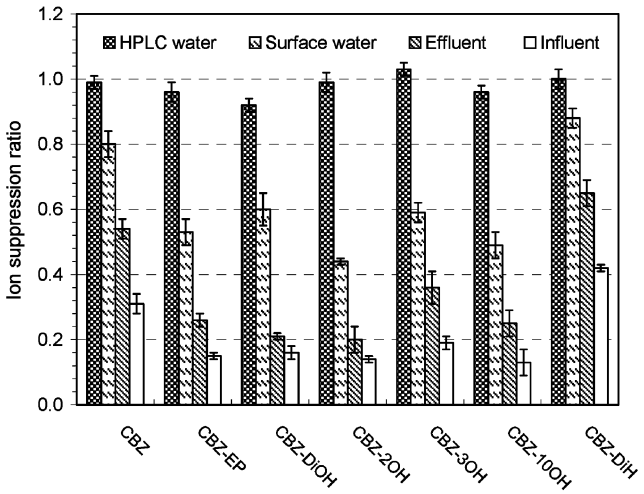


Figure 5. Ion suppression of carbamazepine and its metabolites spiked into different aqueous samples: HPLC-grade water, surface water from Otonabee River, and influent (untreated sewage) and effluent (treated sewage) from the STP of Peterborough, ON.

analytes. There was no ion suppression or enhancement with HPLC-grade water, but ion suppression was observed with surface water. More severe ion suppression occurred with analytes in STP effluent and influent. Only 13−42% of the expected ion signals were observed for the analytes and the internal standard in STP influent. The result indicates that coextracted organic matter in sewage is responsible for severe ion suppression, and lower amounts of organic matter in surface water samples produce less severe ion suppression.

Quantification. Matrix effects definitely affect the quantification of analytes using LC−ES-MS (or MS/MS). The use of isotopically labeled internal standards is preferred in any quantitative mass spectrometric method. However, isotopically labeled standards for carbamazepine and its metabolites were not commercially available. Due to the suitable chromatographic retention time and molecular similarity, dihydrocarbamazepine was used as a surrogate internal standard for analytical quantification. The analytes were quantified in sample extracts relative to the surrogate internal standard, which corrects for losses of analytes during extraction or sample preparation, as well as for variations in instrument response from injection to injection. Five-point calibration curves were developed for analytes across a range of concentrations that are typical for the analytes in aqueous samples. Response factors of analytes relative to the surrogate internal standard were calculated, based on the calibration curves, and these were applied for quantification of the analytes in sample extracts. To ensure accuracy, a calibration curve was developed for each type of matrix sample. The linearity of calibration curves was within $r^2 > 0.999$.

Table 4. Concentrations of Carbamazepine and Its Metabolites in Influent and Effluent Samples from the STP of Peterborough, ON, and Surface Water Samples from the Otonabee River, ON[a]

analyte	influent	effluent	surface water
CBZ	368.9 ± 5.3	426.2 ± 6.1	0.7 ± 0.0
CBZ-EP	47.2 ± 1.8	52.3 ± 1.2	nd[b]
CBZ-DiOH	1571.7 ± 31.0	1325.0 ± 12.2	2.2 ± 0.3
CBZ-2OH	121.0 ± 1.6	132.3 ± 2.1	nd
CBZ-3OH	94.8 ± 2.2	101.5 ± 0.3	nd
CBZ-10OH	8.5 ± 0.6	9.3 ± 0.4	nd

[a] The values represent the mean concentrations and standard deviations of triplicate determinations and are expressed in ng/L. [b] Not detected.

Accuracy and Precision. Accuracy and precision of the method were evaluated by triplicate analyses of the samples. Accuracy was evaluated by determining recoveries of spiked analytes. Known amounts of the analytes were added to the aqueous samples, and the extracts prepared from these spiked samples were then analyzed by LC−MS/MS. The concentration of the analytes in the spiked samples was 100 ng/L. The mean recoveries and analytical precision, as the standard deviations of the mean recoveries, are shown in Table 3. The recoveries ranged from 83.6 to 103.5% in the aqueous samples, with standard deviations of 2.4−5.9%.

Application to Environmental Analysis. To evaluate the SPE and LC−ES-MS/MS analytical method for environmental samples, STP influent and effluent and surface water samples were analyzed. Figure 4 illustrates the chromatogram for an effluent sample from the Peterborough STP, and Table 4 summarizes the concentrations of carbamazepine and its metabolites in the aqueous samples. Carbamazepine is one of the most frequently detected drugs in the aquatic environment in Canada[22] and in Europe. [29,30] In the effluents from 30 STPs in Germany sampled from 1996 to 1998, carbamazepine was detected in all of the STP effluents at a median concentration of 2100 ng/L and a maximum concentration of 6300 ng/L.[29] In the same study, carbamazepine was detected at 24 river and stream sampling sites at a median concentration of 250 ng/L and a maximum concentration of 1100 ng/L. In Switzerland, carbamazepine was detected in lakes, rivers, and STP effluents at concentrations of 35−60, 30−250, and 100−800 ng/L, respectively.[30]

In the samples examined in the work reported here, carbamazepine was detected in STP influent and effluent and in surface water at 368.9, 426.2, and 0.7 ng/L, respectively. The average concentration of carbamazepine in the Peterborough STP effluent lies near to the midpoint of the results from Switzerland, and is lower than those detected in German STPs. These results are consistent with carbamazepine concentrations that we previously reported in STP effluents and surface water in Canada.[22]

(29) Ternes, T. A. *Trends Anal. Chem.* **2001**, *20*, 419−434.
(30) Öllers, S.; Singer, H. P.; Fässler, P.; Müller, S. R. *J. Chromatogr., A* **2001**, *911*, 225−234.

In addition to carbamazepine, Table 4 shows that all five metabolites of carbamazepine were detected in the STP influent and effluent samples, and carbamazepine and 10,11-dihydro-10,11-dihydroxycarbamazepine were detected in surface water from the Otonabee River. The concentrations of 10,11-dihydro-10,11-dihydroxycarbamazepine were ~3 times that of carbamazepine in all of the aqueous samples. For example, 10,11-dihydro-10,11-dihydroxycarbamazepine was detected in the STP influent and effluent at 1571.7 and 1325.0 ng/L, respectively, compared with carbamazepine at 368.9 and 426.2 ng/L in the STP influent and effluent, respectively. In surface water, 10,11-dihydro-10,11-dihydroxycarbamazepine and carbamazepine were detected at concentrations of 2.2 and 0.7 ng/L, respectively. The active metabolite, 10,11-dihydro-10,11-epoxycarbamazepine, was also detected in the influent and effluent samples. The concentrations of carbamazepine and its metabolites in both STP influent and effluent samples are in the order of 10,11-dihydro-10,11-dihydroxycarbamazepine > carbamazepine > 2-hydroxycarbamazepine-3-hydroxycarbamazepine > 10,11-dihydro-10,11-epoxycarbamazepine > 10,11-dihydro-10-hydroxycarbamazepine. Concentrations were similar in the influent and effluent, indicating little removal during sewage treatment.

The major metabolite, 10,11-dihydro-10,11-dihydroxycarbamazepine, was detected at higher concentrations than the parent drug, carbamazepine, as anticipated. With single-agent therapy using carbamazepine, the steady-state concentrations of 10,11-dihydro-10,11-epoxycarbamazepine are ~20−25% of the parent drug. However, when multiple drugs are used, especially when carbamazepine is administered together with valproate or lamotrigine, the 10,11-dihydro-10,11-epoxycarbamazepine concentrations increase significantly to ~50% of the parent drug.[31] In the wastewater samples from the Peterborough STP, 10,11-dihydro-10,11-epoxycarbamazepine concentrations were ~12% of the carbamazepine concentrations. The 10,11-dihydro-10,11-epoxycarbamazepine is biotransformed to the therapeutically inactive product, 10,11-dihydro-10,11-dihydroxycarbamazepine. Other antiepileptic drugs can also generate similar metabolites and thus contribute to the metabolite profile. For example, 10,11-dihydro-10-hydroxycarbamazepine and 10,11-dihydro-10,11-dihydroxycarbamazepine can be generated from a keto analogue of carbamazepine, oxcarbazepine.[32,33] Because 10,11-dihydro-10,11-epoxycarbamazepine is pharmacologically active and is equipotent to the parent drug, more work is warranted to determine the concentrations of this metabolite in STP effluents and adjacent surface water.

Some metabolites of carbamazepine exist in the conjugated forms in urine. The secondary *N*-glucuronide of carbamazepine, *N*-glucuronide of 10,11-dihydro-10,11-epoxycarbamazepine, *O*-glucuronide of *trans*-10,11-dihydroxycarbamazepine, and isomeric *O*-glucuronides of hydroxy-, dihydroxy-, and hydroxymethoxycarbamazepine have been identified as urinary metabolites.[15,34,35] The cleavage of glucuronide conjugates may occur during the STP treatment process, releasing carbamazepine and metabolites into the free forms. Ternes et al.[36] found that the concentrations of free estrogens increased after STP treatment due to the cleavage of estrogen glucuronides. Future work should investigate the fate of conjugated carbamazepine and its metabolites.

CONCLUSIONS

A method was developed for analysis of carbamazepine and its five metabolites, 10,11-dihydro-10,11-epoxycarbamazepine, 10,-11-dihydro-10,11-dihydroxycarbamazepine, 2-hydroxycarbamazepine, 3-hydroxycarbamazepine, and 10,11-dihydro-10-hydroxycarbamazepine in aqueous samples using SPE followed by LC−ES-MS/MS analysis. This is the first method for the determination of carbamazepine and its major metabolites in environmental matrixes. This method will be used to evaluate the removal efficiency for these drugs in STPs and to investigate their distribution in the aquatic environment. The data generated during method validation indicate that the carbamazepine metabolite, 10,11-dihydro-10,11-dihydroxycarbamazepine, is present at higher concentrations than the parent compound in the environment. The active metabolite, 10,11-dihydro-10,11-epoxycarbamazepine was also detected in sewage effluents, but not in samples of surface water near an STP discharge.

ACKNOWLEDGMENT

Financial support was provided by the Natural Sciences and Engineering Research Council (Canada) through its strategic grants program. Novartis Pharma AG (Basel, Switzerland) kindly provided carbamazepine metabolite standards. We thank Drs. Ray March and Jane Mackie for their critical review of the manuscript.

Received for review March 3, 2003. Accepted April 29, 2003.

AC030082K

(31) Shen, S.; Elin, R.; Soldin, S. J. *Clin. Biochem.* **2001**, *34*, 157−158.
(32) Hainzl, D.; Parada, A.; Soares-da-Silva, P. *Epilepsy* **2001**, *44*, 197−206.
(33) Mandrioli, R.; Ghedini, N.; Albani, F.; Kenndler, E.; Raggi, M. A. *J. Chromatogr., B* **2003**, *783*, 253−263.
(34) Lynn, R. K.; Smith, R. G.; Thompson, R. M.; Deinzer, M. L.; Griffin, Gerber, N. *Dru Metab. Dispos.* **1978**, *6*, 494−501.
(35) Maggs, J. L.; Pirmohamed, M.; Kitteringham, N. R.; Park, B. K. *Drug Metab. Dispos.* **1997**, *25*, 275−280.
(36) Ternes, T. A.; Kreckel, P.; Mueller, J. *Sci. Total Environ.* **1999**, *225*, 91−99.