Summer 2014

Detection of the Pharmaceuticals Carbamazepine and Diphenhydramine in Tissue Extracts Using Gas Chromatography-Mass Spectrometry (GC-MS)

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DETECTION OF THE PHARMACEUTICALS CARBAMAZEPINE AND DIPHENHYDRAMINE IN TISSUE EXTRACTS USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

by

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AN UNDERGRADUATE THESIS

Presented to the Faculty of
The Environmental Studies Program at the University of Nebraska- Lincoln
In Partial Fulfillment of Requirements
For the Degree of Bachelor of Science

Major: Environmental Studies
With the Emphasis of: Natural Resources
Minors: Water Science
Environmental Restoration

Under the Supervision of Dr. Daniel Snow

Lincoln, Nebraska

August 2014

Abstract:

The increasing occurrence of pharmaceuticals at low concentration and other "emerging contaminants" in the surface and ground water, has led to a critical need for a wide variety of methods for the accurate measurement of chemicals at low concentrations in a cost effective manner. The objective of this study was to develop a sample preparation method that allows the quantification of two pharmaceuticals (carbamazepine and diphenhydramine) commonly reported in treated municipal wastewater in plant tissue (leafy lettuce) extracts using Gas Chromatography-Mass Spectrometry (GC-MS). Solid phase extraction (SPE) was used to extract analytes while additional sample purification was attempted through the use of reverse phase C18-bonded silica environmental cartridges and polymeric HLB^{TM} cartridges. Sensitivity for the instrumental method was determined to be 36.6ng/g for diphenhydramine and 12.3ng/g for carbamazepine with a standard deviation of 4.0920 and 12.2284, respectively.

Introduction:

Expansion of urban communities throughout the world alongside rapid improvements in analytical detection methods is leading to an increase in detection of pharmaceuticals within waterways (Bartelt-Hunt, et al., 2009). Though detection of pharmaceuticals has been established through several studies (Ferrer, et al., 2004), a lack of research has been applied to the impacts on vegetation and human consumption. Reuse of wastewater is increasingly being explored as a means for conserving water resources. The finite amount of fresh water makes "reclaimed" or "recycled" water a viable option to increase the available supply of water while decreasing the human demand on fresh water (Anderson, 2000).

Pharmaceuticals in recycled water lead to their presence in terrestrial and aquatic environments (Kinney, et al., 2006). Continuous irrigation of reclaimed water containing traces of pharmaceuticals may be a potential route for uptake in vegetables and other crops. Accumulation of pharmaceuticals carbamazepine, diphenhydramine and fluoxetine has been observed in soy beans (Wu, 2010) and antibiotics tetracyclines, tylosin, sulfamthazine, amprolium, monensin, virginiamycin, penicillin and nicarbazine in corn, green beans, and cabbage (Kumar, 2005). The organic and high water content of leafy lettuce, alongside its raw consumption make an ideal target for diphenhydramine and carbamazepine uptake.

Diphenhydramine (2-diphenylmethoxy-N, N-dimethylethanamine) is one of the most popular over-the-counter antihistamines available on the market. It is used to relieve common allergy symptoms such as itching and congestion, but has been used as a short-term sleep aid and to treat motion sickness (Ferrer, et al., 2004). The consistent and constant use of diphenhydramine has lead to an increase in the occurrence of the compound within the environment (Li, 2011). The hydrophobic nature of diphenhydramine increases the potential for detection, even after consumption, excretion and discharge (Ferrer, et al., 2004).

Carbamazepine (5H-dibenzo[b,f]azepine-5-carboxamide) is a common antiepileptic drug poorly removed by routine wastewater treatment facility measures and readily found in surface waters (Ramaswamy, et al., 2010). It is used to treat seizures and mood swings. Since humans exclusively use this compound, the anthropogenic drug is the direct result of human use and excretion (Linden, 2012). Carbamazepine's mode of action is poorly understood and is not completely stable in its current state, resulting in derivatives of the parent compound (Haroune,

et al., 2014).

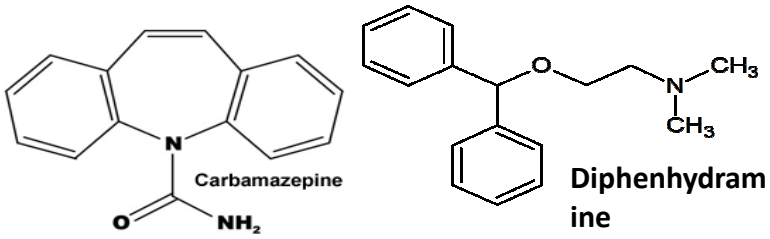


Figure 1: Chemical Structures of Carbamazepine and Diphenhydramine

High-performance liquid chromatography combined with mass spectrometry (LC-MS/MS) is widely used as the analytical instrument of choice for pharmaceuticals due to high efficiency, efficient formation of fragment ions by collision-induced dissociation, and the capability of the mass spectrometer to establish lineage and identity of individual product ions from a selected precursor ion (Ferrer, et al., 2004). The greatest hindrance of LC-MS/MS is higher cost for instrumentation and operation. Thus, creating an effective detection method using a lower cost and more widely available method using GC-MS is desirable not only in the academic community, but also in a commercial setting. Gas chromatography-mass spectrometry (GC-MS) is the analytical method in which a small volatile liquid is changed into gaseous solutes that respond to a detector (Harris, 2009). The mixture of gaseous solutes separates as the gas travels through the column. The separation of these solutes and travel time through the column is known as the retention time and is used to identify each compound. The GC-MS is one of the most widely used analytical techniques used in environmental analysis.

Analysis of carbamazepine and diphenhydramine on GC-MS requires an additional step known as derivatization. This is a technique used to transform the volatility of a compound allowing the reaction to be more reliable, stable, and to have a wider range of substrates. The original compound may exhibit some properties, such as poor volatility, poor thermal stability or

absorption to the injector will yield nonresponsive peak areas, heights and shapes. The low volatility of carbamazepine requires the derivatization of the drug into its derivatives in order to be analyzed on the GC-MS (Javadzadeh, 2007) (Togola & Budzinski, 2007). This process did not impact Diphenhydramine since it is volatile. Derivatization is labor intensive, time consuming and increases the possibility for contamination and errors (Petrovic et. al, 2003).

Materials and Methods:

Experimental

Chemicals and reagents

All solvents were HPLC grade purchased from Fisher Scientific. Carbamzapine-d10 -and orphendadrine were used as internal standards and purchased from Cerilliant and Aldrich, respectively. The surrogate, terbutylazine, was purchased from Sigma Aldrich. The derivatizing agent, N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), and trimethylchlorosilane (TCMS) was purchased from Sigma-Aldrich. Extraction vacuum manifold with Teflon tubing, filter holders and reservoirs were purchased from Sigma-Aldrich. The solid phase extraction (SPE) vacuum manifold and cover was acquired from Sigma-Aldrich. The SPE cartridges, tC18 environmental cartridges, were from Waters Oasis. Borosilicated glass culture tubes were purchased from Fisher Scientific. The block heater and heating block used in the derivatizing reaction is from Lab-Line Instruments. The micro-reaction vessels (1.0 mL) used in the derivatizing process are from Supelco. The GC autosampler vials, seals and inserts are all from Sigma-Aldrich. An analytical balance, capable of weighing to the nearest 0.0001 mg was used.

Solutions

Stock solutions were made to a concentration of 0.1ng/µl for carbamazepine,

carbamazepine-d10, diphenhydramine and orphenadrine. The analyte spike was made to a final concentration of 10ng/ul. The internal standard spike was made to a final concentration of 20ng/µl. The surrogate spike was made to a final concentration of 10ng/µl. MSTFA +1%TCMS was created by placing one vial of 1,000µl MSTFA into a salinized solution vial and spiked with 10µl TCMS.

Sample Collection

Green Star Lettuce (Johnny's Selected Seeds, Winslow, ME) was planted and thinned to a consistency of two representative plants per pot. Six pots formed a tray. Spiking treatments were used on six trays, while three trays acted as a control. Three low treatment trays were continuously irrigated with one liter of a low concentration spike solution (0.5mg/L of carbamazepine and diphenhydramine). The three high treatment trays were continuously irrigated with one liter of a high concentration spiking solution (1.0mg/L of carbamazepine and diphenhydramine). Irrigation occurred three times a week or on an as-needed basis dependent on greenhouse conditions. All treatments and controls were fertilized three times over the span of the six week maturation period. Harvesting was completed after maturation period. The lettuce from all three treatments were collected, weighed and cut into smaller proportions at six week. The treatment representatives were homogenized and stored in plastic bags. The leaf samples were stored in the freezer at -20°F.

Sample preparation and extraction:

5 grams from each representative treatment was taken and measured into a 50mL centrifuge vial. 20mL of methanol was added to each vial and spiked with 100uL of surrogate

spike (20ng/ul). Quality control (QC) samples were spiked with 100uL of 20ng/ul analyte spike. Samples were allowed to seep for 24 hours.

Supernatant was decanted into RapidVap tubes and a 20mL methanol rinse was used to ensure transfer and then decanted into proper RapidVap tubes. The supernatant was placed on the RapidVap for 60 minutes at 60°C under nitrogen gas until dry. The samples were brought up in 100mL of deionized water and spiked with 100uL Internal Standard Spike (20ng/ul).

Samples were run through tc18 environmental cartridges preconditioned with 5mL ethyl acetate, methanol, and deionized water or HLB™ cartridges preconditioned with 3mL methanol and 3mL deionized water. Samples were extracted through cartridges using rinsed Teflon tubing and a glass fiber filter. The samples were extracted at a rate of 3mL/min with a total extraction time of approximately 34 minutes. Once extracted, SPE valves were opened and cartridges were allowed to dry on full vacuum for 5-10 minutes.

Absorbed analytes were eluted from the c18 resin in each cartridge with 4mL of ethyl acetate while HLB™ cartridges were eluted with 3mL methanol and 3mL acetone at a flow rate of approximately 0.5mL/min and captured in 10mL disposable glass culture tubes. Samples were blown down to 1mL and any water was removed via glass pipette and a small scoop of anhydrous Sodium Hydroxide (Na_2SO_4).

Extracts were transferred to acetone washed conical reaction vials with additional ethyl acetate used to rinse test tube to ensure proper transfer. Samples were evaporated to dryness using nitrogen gas. Once reaction vials were dry, derivatization was employed by spiking vials with 100uL MSTFA +1% TCMS, capped with a clean Teflon-lines septa and vortexed. Vials

were then spiked with 100ul pyridine, capped and vortexed. They were then placed on the heating block for 1 hour at 60°C.

After allowing the vials to cool, the derivatized contents were transferred into GC vial inserts and capped with crimping tool. The vials were then analyzed through GC-MS.

Instrumental Analysis

Agilent Technology 5890 Series II Gas Chromatography was coupled with a 5972 MSD. The Injector was a 7673 autosampler and Aligent EnviroQuant ChemStation G17018A Version B.01.00 was used for quantification. This instrument was an open capillary system with split less injection opening at 1.8 minutes.

Injection temperature and detection temperature was set at 280°C. Initial injection of 1µl was assisted by helium through the GC at a flow rate of 1.00mL/min and at a pressure of 7.5psi. The internal temperature was set for 90°C for 2 minutes with a temperature ramp of 15°C per minute. Once 150°C was reached, ramping of 5°C per minute until a temperature of 250°C occurred. A one minute ramp rate of 15°C to a temperature of 275°C was held for 10 minutes then extracted through the column for a total heating time of 37.67 minutes. Samples were passed through an RTX Restex column at a velocity of 35.9cm/sec. The column was 30 meters long with an inner diameter of 0.25mm and a micron film of 0.25mm.

Ion chromatographs (Figure 2) produced by GC-MS are analyzed by abundance and time where abundance refers to the mass of the ion and not a quantification of the compound. For instance, diphenhydramine (DPH) and carbamazepine (CBZ) were both spiked at the same concentration of 4,000ng but the area for diphenhydramine is greater than carbamazepine. This indicates the mass of diphenhydramine's ion is greater than carbamazepine's. Fragment ions for

orphenadrine and diphenhydramine were both detected at m/z=165. Terbutylazine was detected at m/z=214. Carbamazepine-d10 and carbamazepine were detected at m/z=203 and m/z=193 respectively.

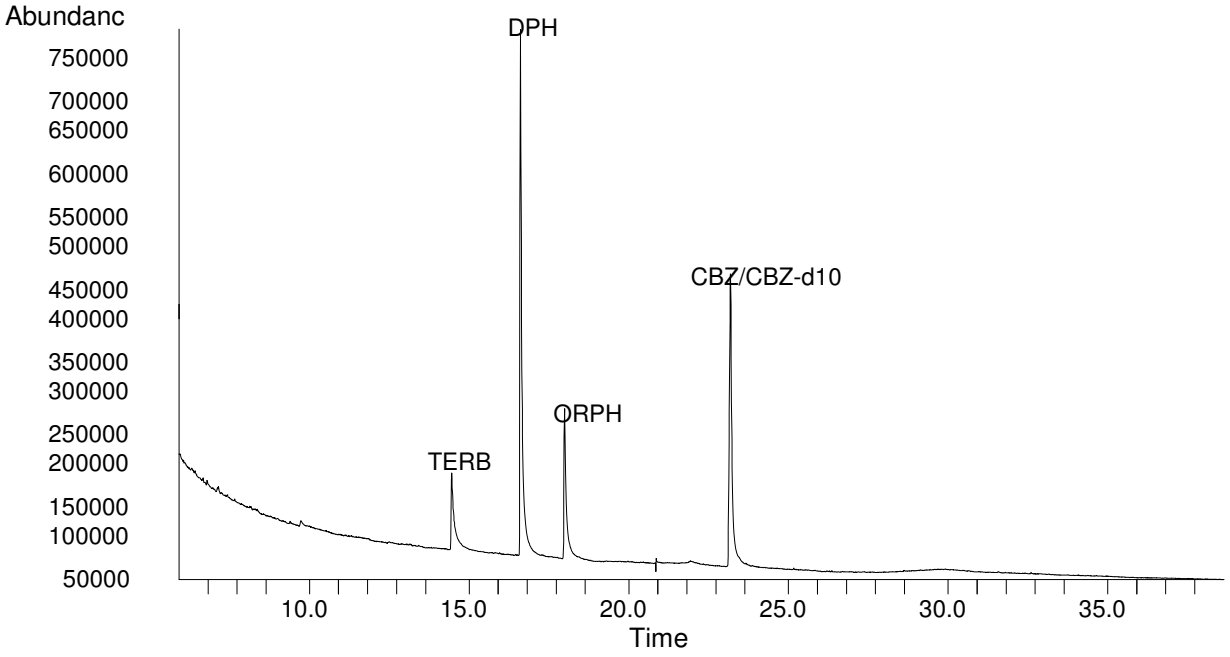


Figure 2: Ion Chromatogram of Calibration 1. Internal Standards were spiked at 2000ng. Analytes and surrogate were spiked at 4000ng.

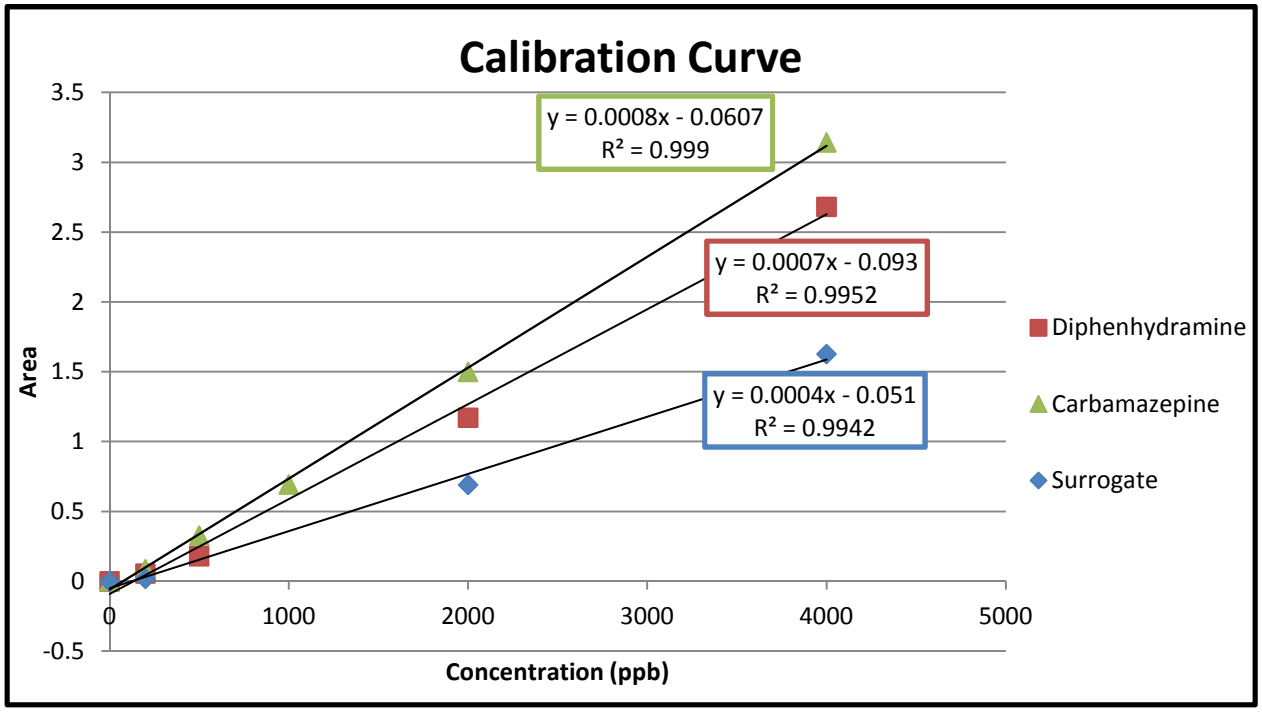
Instrument Detection Limit (IDL) for GC-MS was determined by identifying the smallest signal above the background noise that can be reliably detected. This requires minimal preparation and handling and is performed by duplicating the smallest analyte calibration eight times. The smallest calibration was spiked at an internal standard of 2000ng, analyte spike of 200ng and a surrogate spike of 200ng. The IDL for diphenhydramine was found at 36.661ng/g and for carbamazepine at 12.268ng/g. This indicates the GC-MS can reliably detect these compounds no lower than the given concentrations. The percent recovery for carbamazepine was

69.77% and for diphenhydramine was 79.19%, with a standard deviation of 4.0920 and 12.2284 respectively.

Results

Standard Calibration Curve

The calibration curve is created by plotting the peak area of the GC-MS response versus the concentration of interest. Five standards of known concentration were used to obtain a calibration curve which presents as a linear regression equation. The slope factor of the line is the response of the GC per unit change in concentration. This is known as the calibration sensitivity. The resulting values were in accordance with the standard testing values with the minimum at 0.9942.



C18 Extraction and Recoveries

Variation in extraction from c18 environmental cartridges yielded a high rate of variability in both tests. The first test was a water matrix cartridge elution test with an analyte

spike of 1000ng. Carbamazepine was detected at 115.082ng/g with a standard deviation of 4.72. Diphenhydramine was below detection limit. Recovery for carbamazepine and surrogate was 83.45% and 105.7% respectively. A second test, a full method detection limit (MDL) was performed at a concentration of 200ng to examine the variance levels at a lower concentration. Analysis indicated too high of variation in both carbamazepine (209.046ng/g) and diphenhydramine (145.761ng/g) with a surrogate recovery of 106.85%.

HLB™ Extraction and Recoveries

Hydrophilic-Lipophilic-Balance (HLB™) reverse phase sorbent was used to enhance retention time for polar analytes and low molecular mass (Dias et. al, 2002). HLB™s are often used in pharmaceutical analysis. An elution test of HLB™ cartridges with 200ng of analyte yielded a carbamazepine detection limit of 70.327ng/g with a percent recovery of 87.26% and a standard deviation of 12.042. Detection limit for diphenhydramine was 122.754ng/g with a percent recovery of 115.85% and a standard deviation of 12.042. Surrogate recovery for HLB™ was 67.01%.

Conclusion and Future Perspectives

The complexity of the analysis of diphenhydramine and carbamazepine from leafy green lettuce provided insight for future GC-MS analysis. Though this process did not work as intended, this study could lay a foundation for future analysis.

Though derivatization process is time consumptive and preparation intensive, instrument detection limits were well within the acceptable range for GC-MS. The derivatization of carbamazepine successful produced an IDL of 12.268ng/g with a standard deviation of 4.0920 while diphenhydramine produce an IDL of 12.268ng/g with a standard deviation of 12.2284.

The reverse phase c18-bonded silica environmental cartridges have a strong hydrophobic, lipophillic nature making them ideal for a wide, non-polar range of compounds. The high variance, especially in respect to diphenhydramine, indicates the c18 cartridge was not best suited for this analysis due to the inability of the cartridge to capture polar analytes. The HLB™ cartridges contain resin made from divinybenzene and vinyl pyroolidinone. The pyroolidinone attracts hydrophilic groups and enhances retention for some polar analytes; better suited for the hydrophilic nature of diphenhydramine. The polar nature of the HLB™ reverse phase sorbent enhanced and lowered the variability between compounds, yielding a detection result of 122.754ng/g for diphenhydramine with a standard deviation of 21.020 and 70.327ng/g for carbamazepine with a standard deviation of 12.042.

Future studies may seek to test solvent extractions from an HLB™ cartridge. This study utilized 3mL methanol followed by 3mL of acetone elution. The results suggest two possibilities: analytes may not be attaching to the cartridge during initial SPE extraction or the polarity of the solvent may not be as great as the polarity of the cartridge causing analytes to remain on the cartridge. Low surrogate recoveries may be attributed to improper ion interactions between surrogate and HLB™ cartridge. Future studies of solvent extraction may produce better results and lower detection limits. Use of a different surrogate for analysis on HLB™ may yield better recoveries. To improve time constraints, application of the processes may be best suited for volatile pharmaceuticals, thus eliminating the need for derivatization.

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