Development of an analytical method to extract and detect pharmaceuticals in plant matrices

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Introduction

It has been shown that human-use macrolide antibiotics (azithromycin, clindamycin, and roxithromycin) are environmentally available in wastewaters, source waters, and biosolids. Since some water authorities use the treated wastewater effluent for non-potable water reuse such as for crops, it is important to better understand the fate of these compounds into plants via root migration. In order to achieve that, we developed an analytical method to extract and detect these contaminants in plants grown in soils watered with treated wastewater effluent from a southwestern city (~ 1 million population, July 2008).

A new analytical extraction method had to be developed to extract the antibiotics from the complex matrix of plant samples. In order to verify the validity of the results, the analyzed data, included spiked samples as well as non-spiked samples of each plant collected. The treated wastewater effluent had previously been characterized, and was known to contain the macrolide antibiotic azithromycin, the over-the-counter drug pseudoephedrine, the illicit drug methamphetamine, and an industrial flavoring agent n,n dimethylphenethylamine (n,n'-dmpea, an isobaric compound to methamphetamine).

Study Design

The plants were dissected and separated into leaf and root, then air-dried. The air-dried samples were homogenized and 1-g subsamples were extracted using a modified pressurized liquid extraction (PLE) technique, followed by a rigorous hexane clean-up. Subsequent PLE extracts were analyzed by liquid chromatography-electrospray-ion trap mass spectrometry (LC-ESI-ITMS/MS) in the positive ionization collision induced mode (CID) for greater specificity.

The samples for analysis were taken from two parallel plant uptake studies (I & II), and one biosolids-amended field near study II (study III).

In study I, three crops (lettuce (*Lactuca sativa*), spinach (*Spinacia oleracea*), and carrots (*Daucus carota sativus*), were grown in sand, irrigated with varying concentrations of the two macrolide antibiotics (azithromycin, roxithromycin) and one lincosamide antibiotic (clindamycin). The concentrations were selected relative to concentrations found in waste streams and were 0, 0.1, 1, 10, 100, and 1000 ng/L. After dissolving the antibiotics in methanol we diluted to 1000 ng/L with Colorado River water. All other concentrations were achieved by serial dilutions with Colorado River water.
All crops were sampled at maturity. After harvest, the plants were dissected and separated into leaf and root, then freeze-dried.

In study II, which was conducted at the University of Arizona Tucson research farm field, several crops: lettuce, spinach, carrots, tomatoes (*Lycopersicon esculentum*), peppers (*Capsicum annuum*), melons (*Cucumis melo*) and watermelons (*Citrullus lanatus*))were irrigated with treated wastewater effluent, alongside a well water as a control comparison. All crops were sampled at maturity. After harvest, the plants were dissected and separated into leaf and root, and where appropriate fruit, then freeze-dried.

In study III, bermuda grass hay samples were collected from a field in southern Arizona that had been amended with biosolids from a large (> 3 million pop) for several years. The grasses were dissected and separated into leaf and root, then air-dried.

METHODS/RESULTS

SAMPLE PREPARATION, EXTRACTION AND CLEANUP

Plant and Soil preparation

After collection, plant samples were dried. Crop samples were freeze dried for a week, or longer, until no moisture was present. The dried samples, were then placed into 25 ml zirconium oxide/steel jacketed grinding jars, along with one zirconium oxide grinding ball and are ground using a high impact ball mill (mixer mill 301, Retsch Inc, Newtown, PA) for 3 minutes at a frequency of 20.0 s^{-1}.

Pressurized Liquid Extraction (PLE)

Plant samples were extracted using an Accelerated Solvent Extraction (ASE) system (Model ASE 200 Accelerated Solvent Extractor, Dionex Corporation, Sunnyvale, CA) and 22-mL stainless steel extraction cells. It is necessary to prepare the extraction cell before adding the samples. A glass fiber filter is placed at the bottom of the cell and is covered by approx. 2.5-g alumina, followed by a layer of 2.5-g florosil, then a mixture of 1-g of plant sample, 6-g alumina, and 1-g of hydromatrix is added, followed by hydromatrix filled to top, capped with a glass-fiber filter, and sealed. Two solvent programs are necessary in order to fully extract the analytes. Program one uses a mixture of methyl tert-butyl ether (MTBE):Methanol (90:10), flushed up to 80% of the cell volume, at 50ºC and 1500 psi. After a static period of 15 minutes the eluant is purged into a clean collection vial. Program 2 procedure is with methanol/1%acetic acid, flushed up to 80% of cell volume, at 80ºC and 2800 psi. After a static period of 15 minutes the eluant is purged into a clean collection vial. The MTBE extract is placed into a Turbovap® tube and the extract is allowed decrease by ½ (using a Turbovap® at 4 to 7 psi N_2), then the methanol/acetic acid extract is added to the MTBE extract until a sample volume of 5 mL is reached for plant/root extracts before cleanup is performed.

Cleanup of extracts

Plant and root extracts were washed with hexane, first at a sample volume of 5 ml and then at 1 ml. The number of hexane washes varied from one sample to another, but the washes were done as many times as necessary in order to clean the sample of any undesirable compounds such as chlorophyllic compounds, and waxy materials. The extracts are solvent exchanged with methanol/1% acetic acid and concentrated to 0.5 mL before analysis by LC-ITMS/MS.

MASS SPECTROMETRIC ANALYSIS

Liquid chromatography

The separations were performed using an Ascentis Express C18 (*fused-core technology*) (Supelco-Aldrich, Bellefonte, PA) 2.7 um particle size, 3 cm x 2.1 mm, coupled to a Varian guard column (MetaGuard 2.0 mm Pursuit XRs 3µm C18). Gradient elution conditions were as follows: Mobile phase A 100%, hold for 2 min, 3 min gradient to 30% A:70% B, hold for 5 min, then 3 min gradient to 100% A, hold for 2 min, end run, 5 min equilibration time between analyses. Mobile phase A: de-ionized water/0.5% formic acid; mobile phase B: 82% methanol/18% acetonitrile/0.5% formic acid

HPLC-ESI-ITMS

Mass spectrometric data were acquired with a Varian 500MS (Walnut Creek, CA USA), configured with a liquid chromatograph and an electrospray ion source. The 500MS was run in the positive ionization mode, the voltage applied to the ES needle was approximately 5 kV (dependent upon the optimized response of the ions of interest), the drying gas was set at 20 psi and 200°C, the housing chamber at 50ºC, the nebulizer gas at 40 psi, the spray shield at 600V, and capillary voltages were set dependent upon the optimized response of the product ions of interest. Because of the extremely large amounts of interfering materials co-extracted with the pharmaceuticals, the analyses were performed using the MS/MS mode (collision induced dissociation - CID) for both identification and quantitation of the macrolides and illicit drugs.

Table 1. Emerging contaminants, MW, precursor and product ions, and LODs

Analyte CAS #	Molecular weight (amu)	Precursor ions	Product ion (confirmation ions)	LOD ng, on-column
Azithromycin (83905-01-5)	748.5	749.5 (M+H) $^+$	591.4 (M+H-$C_8H_{16}O_2N$)$^+$)+	0.5
Roxithromycin (80214-83-1)	836.5	859.5 (M+Na) $^+$	755.4 (M+Na-$C_4H_9O_3$)$^+$)+	1
Clarithromycin (81103-11-9)	747.5	748.4 (M+H)$^+$	590.1 (M+H-$C_8H_{16}O_2N$)$^+$)+	1
Clindamycin (18323-44-9)	424.2	425.2 (M+H) $^+$	377.2 (M+H-SH-CH_3)$^+$)+	1
Methamphet-amine (537-46-2)	149.3	150 (M+H) $^+$	119 (M+H-CH_3NH_2)$^+$)+	1.5
MDMA (69610-10-2)	193	194 (M+H) $^+$	163.0 (M-CH_3NH_2+H)$^+$)+	1
Pseudoephedrine (90-82-4)	165.2	166 (M+H)$^+$	148.2 (M+H-H_2O)$^+$)+	0.5
n,n'-DMPEA (1126-71-2)	149.2	150 (M+H) $^+$	105 (M-$N(CH_3)_2$)$^+$)+	0.5

Analytical Challenges

During the development and execution of this methodology for plants we encountered various analytical difficulties, both in the extraction phase and the detection phase.

Extraction difficulties

•Waxy and fatty materials are co-extracted from root matrices, and are not fully removed during hexane washes.

•Numerous hexane (n = 3 to 6) washes are required to remove chlorophyllic and waxy materials from leafy and root extracts.

Detection challenges

•Injection of plant and root extracts build up deposits on spray shield, causing loss of sensitivity, necessitating cleanup of spray shield after every 2nd injection of sample extracts.

•Injection of some plant and root extracts temporarily bind to the column, even with guard column, causing non-detects. This necessitates reverse-flow of high organic solvents then water through the column for cleanup before use again.

RESULTS

Table 2. Accuracy and precision spiked recovery parameters of antibiotics (0.5 µg g^{-1}) from lettuce, spinach, and carrots (roots and shoots).

	% Recovery (standard deviation; % relative standard deviation) †						
Sample type Compound	Bermuda Roots	Lettuce leaf	Lettuce root	Spinach leaf	Spinach root	Carrot root	Carrot tops
AZI	20 (± 4; 20%)	22 (± 2; 10%)	2 (± 1)	45 (± 9; 20%)	5 (± 1; 20%)	19 (± 6; 32%)	19 (± 1; 5%)
RXY	40 (± 3; 8%)	32 (± 5; 16%)	26 (± 2)	29 (± 4; 14%)	48 (± 4; 8%)	76 (± 17; 23%)	35 (± 5; 13%)
CLA	22 (± 6; 25%)	20 (± 2; 11%)	10 (± 1)	22 (± 4; 20%)	16 (± 3; 17%)	32 (± 9; 28%)	21 (± 3; 12%)
CLI	33 (± 7; 22%)	30 (± 8; 26%)	22 (± 1)	23 (± 6; 26%)	38 (± 9; 24%)	35 (± 5; 15%)	32 (± 4; 12%)
METH	44 (± 6; 14%)	24 (± 4; 16%)	15 (± 0)	21 (± 2; 7%)	33 (± 9; 28%)	30 (± 4; 15%)	36 (± 5; 13%)
MDMA	45 (± 8; 17%)	23 (± 1; 6%)	11(± 0)	23 (± 4; 18%)	22 (± 15; 69%)	26 (± 6; 21%)	26 (± 1; 4%)
n,n'-DMPEA	47 (± 10; 21%)	29 (± 1; 5%)	17 (± 2)	22 (± 3; 13%)	23 (± 6; 28%)	29 (± 5; 16%)	38 (± 1; 2%)
PSEUDO	50 (± 3; 6%)	27 (± 0; 0%)	17 (± 1)	24 (± 2; 8%)	20 (± 15; 74%)	23 (± 6; 28%)	28 (± 1; 4%)

AZI = azithromycin, RXY= roxithromycin, CLA= clarithromycin, CLI= clindamycin, METH= methamphetamine, MDMA= ecstasy, n,n'-DMPEA = n,n'-dimethylphenyethylamine, PSEUDO= pseudoephedrine, †n=3 for all sample types, except lettuce roots: n= 2; and carrot roots: n=6

Table 3. Results Phase I - Greenhouse Study†

	ng/g , n = 2					
Spiked Compound	Lettuce leaf	Lettuce root	Spinach leaf	Spinach root	Carrot greens	Carrot root
AZI	ND	ND	ND	ND	ND	ND
RXY	ND	< 10 ng/g LOQ	ND	ND	ND	115
CLI	ND	< 10 ng/g LOQ	ND	< 10 ng/g LOQ*	ND	53

AZI = azithromycin, RXY= roxithromycin, CLI= clindamycin, *not enough sample for duplicate extraction. † All samples are from the 1000 ng/L watered plots.

Table 4. Phase II and III – UA-CAC field study & UA-YAC field study

	ng/g						
	Bermuda grass	Bermuda roots n=2	Carrot Roots n = 2	Green bell pepper n=1	Cantaloupe n = 2	Watermelon n=4	Spinach n=2
n,n'-DMPEA	ND	125	ND	58	53	180	48
Azithromycin	ND	90	ND	ND	ND	ND	ND
Clarithromycin	ND	135	ND	ND	ND	ND	ND

n,n'-DMPEA = n,n'-dimethylphenyethylamine, ND = not detected

CONCLUSIONS

- We detected no uptake of azithromycin in any of the plant/root samples from either the greenhouse or Tucson effluent field crops.
- There were traces of uptake of clindamycin into the spinach roots and lettuce roots, however we did not have enough root sample to perform a duplicate extraction/analysis.
- Trace amounts of roxithromycin were detected in lettuce roots. Carrots showed the greatest amount of uptake of roxithromycin, 115 ng/g, and clindamycin, 53 ng/g, from the 1000 ng/L watered into the carrot plots. No compounds were detected in the lower level watered plots.
- All of the plants, except the carrots, from the field crops watered with Tucson wastewater effluent showed uptake of n,n'DMPEA, an industrial chemical used in manufacturing, food industry, etc.
- The bermuda roots showed uptake of azithromycin, clarithromycin (another macrolide), and n,n'DMPEA.