

# Determination of Pharmaceutical Residues in Wastewater by Immunoassay

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## Abstract

According to a recent USGS study, pharmaceutical and other contaminants from treated municipal wastewater can travel into shallow groundwater following release to streams or other bodies of water. Competitive enzyme-linked immunosorbent assays (ELISA) for detection of Fexofenadine (FEX), Ethynyl estradiol (EE-2) and Cotinine (COT) in wastewater were developed. Rabbit polyclonal antibodies were produced and used for assay development. Total time for each assay was less than 70 minutes. The ELISA lower limit of detection for FEX is 1 ng/ml (ppb), EE-2 is 0.3 ppb and for COT it is 3 ppb. Random samples of influent and effluent wastewater were tested for the presence of these 3 residues. Both influent and effluent wastewater contained FEX residues at low ppb levels. FEX levels in wastewater samples were confirmed by HPLC after purification and concentration. The recoveries of spiked FEX in water was determined and compared by ELISA and HPLC, these were found to be on average 86% and 90% respectively. The method recovery of EE-2 from spiked samples was 138% and COT was 97%. The results indicate that these immunoassays can be used as a rapid and accurate tools for the determination of FEX, EE-2 and COT in environmental water samples.

## Introduction

Treated wastewater and its characteristics have been an important topic to those interested in using this recycled water to irrigate crops. Increasingly arid countries have been using treated municipal wastewater before it has returned to streams, for this purpose. There have been concerns about many types of residues present in the effluent water after processing, and the long term impact of residues (antibiotics, hormones, and other pharmaceuticals) on the environment is not well studied.

ELISA methods were used to quantify levels of three compounds in the influent and effluent wastewater from municipal wastewater treatment plants. Fexofenadine, an over-the-counter antihistamine, was found to be prevalent in wastewater. Ethynyl estradiol and cotinine tests also used wastewater as the test sample with appropriate results. These results indicate that ELISA technology is an easy method to use in detecting residues of many classifications. These tests could be used as a tool to monitor the efficiency of municipal water treatment facilities and the characteristics of discharged water into the environment.

## Methodology

### Polyclonal antibody and test kit production:

In the development of these assays FEX, EE-2, and COT were each coupled to a protein carrier. This protein conjugate served as the immunogen for antibody development in rabbits. The specific antibodies were produced through the immunization of New Zealand white rabbits and the antiserum was collected and tested monthly. Horse radish peroxidase (HRP) was linked to each compound to serve as the enzyme conjugate provided in the test kit. The ELISA test kits are provided in a competitive format with soluble antibody and HRP labeled conjugate. The result is determined by measurement of the absorbance using a plate reader. The color intensity in the developed plate well is inversely proportional to the amount of analyte present in the sample.

## Methodology

### Sample Preparation for FEX Confirmatory Analysis by HPLC:

1. Apply 250 ml wastewater to Strata-X33 U Polymeric Reversed column for purification following the instructions. Wastewater was provided by Water Resource Recovery Division of Saco, Maine.
2. Elute the column with 5 mL methanol and bring to dryness using nitrogen gas.
3. Add 1 ml HPLC Mobil phase to re-suspend the sample.
4. Filter 1 ml sample with a 0.2 µm filter.
5. Load 1 ml sample on HPLC.
6. Compare elution profile of FEX Standard with sample extract.

### HPLC Conditions

- Mobile phase: Ammonium Acetate/ACN (70/30) pH 5.0
- Column: ZORBAX Eclipse XDB-C18 (150 X 4.6 mm, 5 µm)
- Flow rate: 1 mL/min
- Detector: UV 265 nm

### FEX, EE-2, & COT Assay Protocol<sup>1</sup>

1. Add 50 µl of HRP conjugate to each well.
2. Add 50 µl of calibrator or sample.\*
3. Add 50 µl of antibody solution to the wells and mix gently for 30 seconds.
4. Incubate the wells for 30 minutes at room temperature.
5. Dump the well contents and wash 4 times with tap water.
6. Add 100 µl of substrate solution into each well and incubate for 30 minutes at room temperature.
7. Add 100 µl of stop solution and measure absorbance at 450 nm – 650 nm.
8. The concentration is calculated from the calibration curve.

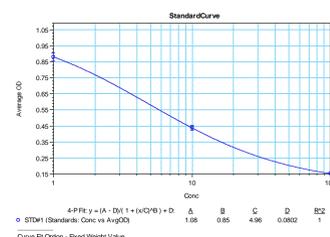
<sup>1</sup> Each ELISA uses reagents specific to the analyte.

\* Wastewater samples are directly used in the assay protocol without modification or extraction.

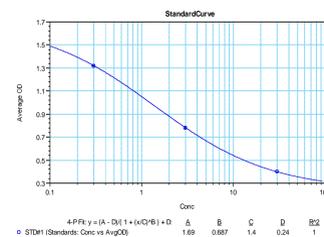
## Results

Table 1. Detecting Pharmaceutical Residues in Wastewater

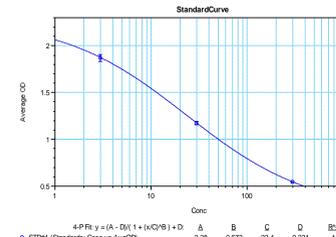
FEX ELISA Effluent Wastewater Samples Tested	Avg. Conc. FEX (ppb)	EE-2 ELISA Effluent Wastewater Samples Tested	Avg. Conc. EE-2 (ppb)	COT ELISA Wastewater Samples Tested	Avg. Conc. Cotinine (ppb)	
					Influent	Effluent
#1	3.6	#1	<0.3	#1	9.4	0
#2	4.1	#2	<0.3	#2	8.9	0
#3	4.0	#3	0.3	#3	8.6	0
#4	3.5	#4	0.3	#4	9.0	0
#5	3.2	#5	<0.3	#5	11.1	0



FEX (ppb)  
Calibration curve (R<sup>2</sup> = 1)



EE-2 (ppb)  
Calibration curve (R<sup>2</sup> = 1)



COT (ppb)  
Calibration curve (R<sup>2</sup> = 1)

Table 2. Spike and Recovery Data for 3 Pharmaceuticals in Wastewater.

Wastewater Sample	Spike (ppb)	FEX ELISA	
		Fexofenadine (ppb)	Recovery (%)
#1	0	2.3	---
	3.0	4.8	83
	6.0	7.8	92
	12.0	11.1	73
#2	0	3.1	---
	3.0	5.7	87
	6.0	8.8	95
	12.0	13.4	86

Wastewater sample	Spike (ppb)	EE-2 ELISA	
		EE-2 (ppb)	Recovery (%)
#1	0	<0.3	---
	10.0	13.8	138
Wastewater sample	Spike (ppb)	Cotinine ELISA	
		Cotinine (ppb)	Recovery (%)
#2	0	<3.0	---
	10.0	9.7	97

## Results

Table 3. Recovery Results from a Fexofenadine spiked sample analyzed by the HPLC confirmation procedure

Sample	Spike (ppb)	HPLC	
		Fexofenadine (ppb)	Recovery (%)
Waste water	0	0.9	---
	3.00	3.6	90

## Discussion

Three separate competitive immunoassays were developed for determination of pharmaceuticals in municipal wastewater samples. These assays are easy to perform and the samples are tested directly without any sample preparation or extraction steps.

FEX was found at low levels (ppb) in both the influent and effluent wastewater samples tested. This was confirmed by HPLC coupled with a purification and concentration step.

The ELISA for the detection of EE-2 can quantify samples containing residues from 0.3 ppb to 30 ppb without dilution. Most of the water samples tested (influent or effluent) contained very low levels of EE-2 residues.

ELISA Test Kits for COT, the predominant metabolite of nicotine in humans, are available now with a quantitative range of detection from 3 ppb to 300 ppb. COT levels in influent wastewater were found to be about 10 ppb, but were not detected in the effluent samples.

These results indicate that the Competitive Enzyme-Linked Immunosorbent Assay (ELISA) kits developed in this study (96-well plate format) can be used as a rapid and accurate tools for the determination of pharmaceutical residues in water samples.

## References

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