Int J Pharma Res Health Sci. 2018; 6 (4): 2701-05
DOI:10.21276/ijprhs.2018.04.10
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CODEN (USA)-IJPRUR, e-ISSN: 2348-6465

PHS Scientific House
PHARMAHEALTHSCIENCES

International Journal of Pharma Research and Health Sciences

Available online at www.pharmahealthsciences.net

Original Article

An HPLC-UV and Fluorescence Method for the Detection of Three Pharmaceuticals in Water Systems

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ARTICLE INFO

Received:28 Jun 2018
Accepted:14 July 2018

ABSTRACT

A new, fast and economical HPLC method was developed for the analysis of carbamazepine, fluoxetine, and venlafaxine in water samples. A reverse-phase HPLC assay was used with UV-Vis and fluorescence detectors. Samples were passed through Gemini C18-110A (250 x 4.60 mm, 5 µm) column at a flow rate of 1.0 ml/min. From spiking experiments, limit of detection (lods) and limit of quantification (loqs) for carbamazepine was 0.01 µg/L and 0.1 µg/L, for fluoxetine were 1 µg/L and 0.1 µg/L and for venlafaxine were 1 µg/L and 0.1µg/L, respectively. HPLC can be used to detect the trace amount of pharmaceuticals in water. The technique requires no derivatization steps, requires less time and is more cost-effective.

Keywords: HPLC, solid phase extraction, carbamazepine, fluoxetine, venlafaxine.

1. INTRODUCTION

Pharmaceuticals in water are considered as a major emerging pollutant because of their ubiquity in the aquatic environment and their negative health effects [1-3]. Conventional wastewater treatment plants (WWTPs) are not specifically designed to remove pharmaceuticals from the wastewater [4]. Moreover, the concentration of pharmaceuticals is very low (50 ng/L to 100 ng/L), hence not much special attention is given to the problem [4]. However, the above-shown facts could be alarming

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especially in the worst-case scenario such as; 1: area close to poorly controlled manufacturing or production facilities, 2: ubiquitous use of a particular pharmaceutical in a specified area, 3: area close to poorly controlled hospital waste management facilities. In addition, individuals such as patients, pregnant women, and fetus are more susceptible to the pharmaceutically contaminated water [5]. Liquid chromatography-mass spectroscopy (LC-MS or MS-MS), gas chromatography-mass spectrometry (GC-MS or MS-MS) following solid-phase extraction (SPE) are the most common and accurate techniques used for the pharmaceutical detection [6]. These sophisticated techniques are expensive, time consuming, and require high degree of analytical knowledge. High-performance liquid chromatography (HPLC) with ultraviolet (UV) or fluorescence detection has been also used for the identification and determination of pharmaceuticals in the water system. Application of improved sample clean up method and quantification by internal standard or standard addition method make the above method more precise [7, 8].

In the current work,a simple, fast and economical HPLC method was developed for the analysis of carbamazepine, fluoxetine, and venlafaxine. The major significance of the developed analytical method is that it can be used for the routine analysis of wastewater in WWTPs. The method is more cost-effective; hence, it can become more widely adopted. Moreover, the current method will help in determining the potential dosage of pharmaceuticals consumed by humans through the drinking water.

2. MATERIALS AND METHODS

Materials

The molecular structure and properties of the pharmaceuticals considered in this study are shown in Table 1. The pharmaceuticals studied were: carbamazepine (Sigma-Aldrich, St. Louis, MO), venlafaxine (TCI, St. Portland, OR) and fluoxetine (TCI, St. Portland, OR) and were of analytical grade (>99%). For HPLC mobile phase HPLC grade acetonitrile (Fisher Scientific, Fair Lawn, NJ), HPLC grade methanol (Fisher Scientific, Fair Lawn, NJ), citric acid monohydrate (Sigma-Aldrich, St. Louis, MO), sodium hydroxide (Mallinckrodt, St. Louis, MO) and ethylenediaminetetraacetic acid (EDTA) (Calbiochem, La Jolla, CA) were used. Ultrapure water from Barnstead International purification system (Barnstead International, Dubuque, IA) was used for distilled water (DI) preparation. SPE cartridges (Oasis HLB, 30 µm)were fromWaters Corporation, Milford, MA.Following instruments were used for the analysis: Pump: SP 8000 ternary HPLC Pump, (Spectra Physics, San Jose, CA), HPLC column: Gemini C_{18} 110A (250 x 4.60 mm, 5 µm) Column (Phenomenex). SP 8450 UV/Vis Detector (Spectra Physics, San Jose, CA) and HP 1046 A (Hewlett Packard) fluorescence detector were used for the detection of the pharmaceuticals.

Method

Preparation of reagents and solutions

1: Preparation of mobile phase: A mixture of citric acid (100 mM) and EDTA (10 mM) was mixed (pH adjusted at 4.5 by using 0.1 M NaOH) in water and was used as solvent A. Mobile phase was made from the solvent A and methanol (20:80, v/v). It was filtered by a 0.22 µm nylon membrane filters and was degassed with helium prior to use.

2: Preparation of stock solution: Stock solutions of fluoxetine and venlafaxine were prepared in water (10 mg/50 mL). However, the stock solution of carbamazepine was prepared in acetonitrile (10 mg/50 mL) because of its low solubility in water. Prepared solutions were stored in dark at -20°C. The stock solution was used for the calibration standards and quality control of the method. Working aqueous solutions were prepared daily. Composite working standard solutions of the pharmaceuticals were prepared periodically by mixing suitable aliquots of the stock solutions diluted with water/acetonitrile (50/50, v/v) and the stored at 4°C.

3: Preparation of sample solution: Sample solution were prepared by diluting all three stock solutions in water to a concentration of 100, 50, 25, 20, 12.5, 6.25, 3.125, 2, 0.2, 0.02 and 0.002 µg/mL. The concentration of the sample solution was calculated from the chromatogram of the standard solution. All the stock solutions (50 mL) and sample solutions (1.5 mL) were stored in aliquots at 4^0 C.

Extraction procedure

To optimize the SPE, aqueous solutions of ultrapure water with a known amount of pharmaceutical was passed through the SPE and varying the activation and elution conditions of the cartridges. To get the best recovery of the analytes, it was crucial to completely dry the solid phase before elution, removing from vacuum after 45 min the residual water from previous washing. No chromatographic interference was detected when analyzing the blank extracts. Isolation of pharmaceuticals from the water samples was done by using SPE cartridge on a VacElut apparatus. First, the cartridge was activated by passing 5 mL of methanol. Subsequently, 1 L of the water sample containing each of the three pharmaceuticals was passed through a Teflon tube at a flow rate of 3 mL/min, using a Supelco 12-port vacuum manifold system (Bellefonte, PA, USA) connected to a vacuum pump. The loaded cartridge was eluted by passing 1 mL of methanol (three 1 mLaliquots) at a flow rate of 3 mL/min. The combined aliquots were evaporated to dryness under a stream of nitrogen. The residue left was dissolved in 300 µL of methanol so that 100 µL of 3 injections can be done in HPLC.

Chromatographic analysis

HPLC was carried with an isocratic elution (20:80) of mobile phase comprising of citric acid (100 mM), EDTA (10 mM) and methanol adjusted to the pH of 4.5 and with a flow rate of 1mL/min. Gemini C18-110A (250 x 4.60 mm, 5 µm particles) column was used and was equilibrated for 30-40

min. with mobile phase before making an injection. The injection volume was set up to 100 µL, column temperature was maintained at 25^0 C and a post-run equilibrium time of 3 min. was used. Carbamazepine was detected by using UV-Vis detector at a set wavelength of 285 nm. Fluoxetine and venlafaxine were detected by using fluorescence detector, with an excitation and emission wavelength of 230 and 300 nm, respectively. Pharmaceuticals were identified by comparing the retention time of the peaks with that of standard solutions. UV-spectra of the peaks in the standard solution and sample solution chromatogram were used to confirm the pharmaceuticals.

Calibration

According to the International Conference on Harmonization guidelines (ICH, 2005), method validation was done by evaluating linearity, specificity, limit of detection (LOD) and limit of quantification (LOQ), accuracy, repeatability and reproducibility, robustness and system suitability. Calibration standards were prepared in concentration from 0.01 to 2.0 µg/mL for all the pharmaceuticals, to cover the concentration range expected for each pharmaceutical in environmental water. To get the external calibration curves, linear regression of peak areas of the standard solution versus their respective concentration was plotted. Linearity was tested with the standard mixture at different concentrations. The correlation coefficient varied in a narrow range from r=0.989 for carbamazepine to r=0.999 for venlafaxine. Linear regression of peak area of standards solutions against the respective concentrations was used to prepare the calibration curve. System suitability test was performed to evaluate the chromatographic parameters (capacity factor, number of theoretical plates, asymmetry of the peaks and resolution between two consecutive peaks) before each validation run. The system suitability criterion is a resolution between the three pharmaceuticals and standard (caffeine) and peaks. The estimation of the LOD and LOQ was done by injecting standard solution serially diluted until the signal-to-noise ratio for LOD was 10:1 and for LOQ was 3:1.Evaluation of the method precision was done by intra- and inter-day repeatability method. For the intraday repeatability, three replicates of spiked water samples using the same equipment and same analytical procedure in 1 day was done.

3. RESULTS AND DISCUSSION

In most of the cases WWTPs are major contributors of pharmaceuticals in the aquatic environment, since important loads are discharged into river waters through effluents wastewaters. Modern WWTP are efficient to remove carbon and nitrogen, as well as microbial pollution control. However, these installations receive also a large number of different trace organic polluting compounds, among them pharmaceuticals, for which conventional treatment technologies have not been specifically designed. Pharmaceuticals may occur in WWTP effluents because they do not have or have low tendency to adsorb onto activated sludge or because their microbial degradation was not fast enough to be completed within the hydraulic retention time of the plants. [9]

Most of the pharmaceuticals, do not currently have stabilized drinking-water standards or health advisories; therefore, the potential health consequences associated with exposure through drinking water are unknown. The concentrations in the efflux water of WWTPs designed for human consumption are far below the concentration used in therapy. For example, the maximum possible intake of carbamazepine in finished water in a lifetime was 13 mg, on the other side a single therapeutic dose of carbamazepine generally is 100 mg or greater. Moreover, most of the studies on the therapeutic effects of drugs are based on the short-term ingestion of relative high doses; very less is known about potential health effects associated with long term chronic ingestion of low concentrations through drinking water [9]. Moreover, the criteria of drinking-water are currently based on the toxicity of individual compounds and not combinations of compounds. Research have shown that exposure to multiple organic compounds, even at low concentrations, may have a synergistic human health consequence is an area of recent research, and the co-occurrence of organic compounds in drinking-water supplies has recently been documented [10].

HPLC with UV and fluorescence detection was chosen as a simple, fast, and effective separation method for the determination of CBZ, venlafexine and fluorextine. In extensive preliminary experiments, a series of aqueous mobile phases with different pH values were tested. Best results were obtained when using solvent A (citric acid, EDTA, NaOH in water) and methanol (20:80, v/v) and adjusting the pH of the solution to 4.5, allowing adequate separation of the drug and the internal standard using a Gemini C_{18} 110A column at a flow-rate of 1.0 ml/min. In the current work venlafaxine and fluoxetine were detected by fluorescence detector whereas carbamazepine was detected by UV/Vis detector. The performance of the SPE-HPLC was characterized by validation procedure with spiked water samples. The analysis was validated by performing three-sample analysis of each concentration. Detection of carbamazepine was done at 10µg/L, 1 µg/L, 0.1 µg/L and 0.01 µg/L concentration with a retention time of 3.78, 3.71, 3.76 and 3.78 minutes respectively. Detection of fluoxetine was done at 10 µg/L, 1 µg/L and 0.1 µg/L concentration with a retention time of 2.71, 2.71 and 2.73 minutes respectively. Finally, detection of venlafaxine was done at 10 µg/L, 1 µg/L and 0.1 µg/L concentration with a retention time of 2.98, 2.97 and 2.95 minutes, respectively. Simultaneous detection of fluoxetine and venlafaxine was done at 10µg/L, 1 µg/L and 0.1µg/L concentration with a retention time of 2.71, 2.73 and 2.71 minutes for fluoxetine and 2.99, 3.05 and 3.0 minutes for venlafaxine. LOD and LOQ for carbamazepine were 0.01 µg/L and 0.1 µg/L, for

fluoxetine were 1 µg/L and 0.1µg/L, and for venlafaxine were 1 µg/L and 0.1 µg/L, respectively. The retention time and LOQ are shown in Table 2. Chromatogram of the three pharmaceuticals is shown in Figure 1.

Table 1:Physical properties of carbamazepine, fluoxetine and venlafaxine.

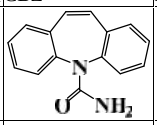
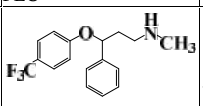
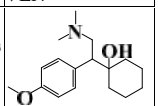
Compound	Carbamazepine	Fluoxetine	Venlafaxine
Abbreviation	CBZ	FLU	VEN
Chemical structure			
Water solubility (g/L) (25 ^{0}C)	0.018	0.014	0.270
Half-life	36 hours (single dose), 16-24 hours (repeated dosing)	1–3 days (acute) 4–6 days (chronic)	5±2 hours (parent compound for immediate release preparations), 15±6 hours (parent compound for extended release preparations), 11±2 hours (active metabolite)
n-Octane/water partition coefficient (log K_{ow})	1.624	2.080	3.280
Henry's law constant at 25^{0}C (atm m^3 mol^{-1})	1.08 X 10^{-10}	8.90 X 10^{-8}	2.04 X 10^{-11}
Excretion in urine (% of the dose)	Urine (72%), faeces (28%)	Urine (80%), faeces (15%)	Ren (87%; 5% as unchanged drug; 29% as desvenlafaxine and 53% as other metabolites)

Table 2: Retention time of carbamazepine, fluoxetine and venlafaxine at different concentrations.

	10µg	1µg	0.1µg	0.01µg	Detector used
Carbamazepine	3.78 min	3.71 min	3.76 min	3.78 min	UV-Vis detector
Fluoxetine	2.71 min	2.71 min	2.73 min	-	Fluorescence
Venlafaxine	2.98 min	2.97 min	2.95 min	-	Fluorescence
Fluoxetine and Venlafaxine	2.71 min 2.99 min	2.73 min 3.05 min	2.71 min 3.0 min	-	Fluorescence

Figure 1: Pharmaceuticals chromatogram: a: carbamazepine, b: fluoxetine, c:venlafaxine,d: venlafaxine and fluoxetine.

Most of the pharmaceuticals get degraded in the environment. However, the degradation of the current pharmaceuticals is very slow because of their complex structures. Moreover, the presence of a double bond makes them harder to be degraded. Babic et al. have used the SPE-HPLC-DAD method for the detection of sulfadiazine, sulfamethazine, sulfaguanidine, oxytetracycline, trimethoprim, enrofloxacine and penicillin G/procaine in the wastewater matrix [11]. Here they have obtained the LOQ of 1.5-100 µg/L. Santos et al. have used HPLC with DAD and fluorescence detector for the determination of pharmaceutically active compounds in wastewater sample [12]. The method is used for the determination of pharmaceuticals such as diclofenac, ketoprofen, acetaminophen, carbamazepine, caffeine (by DAD) and naproxen, and ibuprofen (by fluorescence detection). They have obtained the LOQ in the range of 6.2-319.8 and 3.0-160.0 ng/mL for the influent and effluent wastewater samples respectively. The obtained LOQ by Babic et al. and Santos et al. were lower than our LOQ because water samples in our study were from a clean water source and did not show any matrix effect.

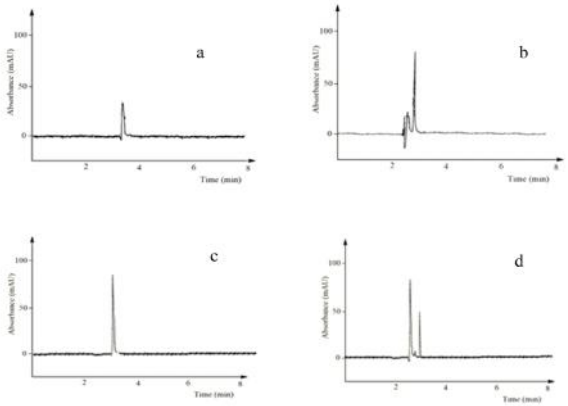


Fig 1: Pharmaceuticals chromatogram: a: carbamazepine, b: fluoxetine, c: venlafaxine, d: venlafaxine and fluoxetine.

The current work is only focused on the detection of the pharmaceuticals in water that are present in the aquatic environment, but in the aquatic environment, pharmaceuticals are present as mixture of a great variety of therapeutic classes, which should be taken into account. There is a need for more advanced water treatments technology, such as ozone oxidation, as the conventional techniques (flocculation, sedimentation, and filtration) are unable to serve the purpose efficiently [13].

The technology to analyze for all known organic compounds is currently unavailable and, therefore, the complete extent of occurrence of OWCs in drinking-water supplies is unknown. The challenge for future studies is to develop the means to characterize the types and concentrations of these compounds that are likely to co-occur in drinking-water supplies and to assess their potential effects.

4. CONCLUSION

A fast, sensitive, accurate and cost-effective HPLC-UV and fluorescence method was developed. The method was used for the detection of three pharmaceuticals: carbamazepine, fluoxetine, and venlafaxine. Use of SPE and HPLC made it a cost-effective and hence an alternate to GC-MS and LC-MS methods. With the current method, we were able to obtain the LOD of 0.01 µg/L, 0.1 µg/L, 0.1 µg/L and LOQ of 0.1 µg/L, 1 µg/L, 1 µg/L for carbamazepine, fluoxetine and venlafaxine, respectively. The current method can perform the routine analysis of the pharmaceuticals discharged from

the WWTPs and can be used to evaluate the performance of the WWTP. Further work is needed to develop cost-effective HPLC methods for the determination of pharmaceuticals and their metabolites in the environment such as surface water, groundwater, and drinking water.

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Conflict of Interest: None
Source of Funding: Nil