IOSR Journal of Engineering
May. 2012, Vol. 2(5) pp: 984-989

Analysis and occurrence of residues of the hormones estriol, 17α-ethinylestradiol and 17β-estradiol in urban water supply by HPLC-DAD

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ABSTRACT

Hormones 17α-ethinylestradiol, 17β-estradiol and estriol were analyzed in urban water supply due to the concern about the problem associated with the presence of these compounds in water bodies. The aims of this project were adapt, validate analytical methodology and monitor these hormones in drinking water. Results showed that the limits of detection, linearity, regression coefficients and recoveries were suitable for the method and no contamination was detected in samples analyzed.

Keywords **- drinking water estriol, 17β-estradiol, 17α-ethynilestradiol, liquid chromatography**

1. INTRODUCTION

Hormones are considered emerging micropollutants [1] and the environmental unbalance caused by these hormones, their distribution and behavior in different environmental levels have had great relevance recently. The introduction of these drugs to the environment stems from a combination of several factors, such as: the quantity produced; the strength (quantity, frequency and duration); efficiency of excretion of conjugated compounds and their metabolites [2]; capacity of adsorption/desorption in soil and metabolic decay in water treatment. The amount of drugs and metabolites incorporated into the environment are low, however their continuous disposal into the environment increases their concentration over time [3] and may cause adverse effects on aquatic and terrestrial organisms [4].

These residual hormones have persistent physical-chemical properties, are lipophilic, bioacumulative and have low pressure steam, facilitating their spread in environment [1,5] and are also considered endocrine disruptors [6]. Because some endocrine disruptors are fat soluble, high concentrations of these compounds can accumulate in meat, fish, eggs and dairy foods [7,8].

The hormone 17β-estradiol (E2), used in hormonal replacement therapies [5], for example, was found in several types of meat (beef, pork, poultry, fish), milk and dairy products, eggs and plants (grasses and vegetables) [9]. The E2 and estrone (E1) were also found in aquatic organisms (at low concentrations from μg L^{-1} to ng L^{-1}). These drugs are designed to keep persistent properties, and a proportion of the drug from 50 – 90% is excreted unaffected into the sewage, remaining in the environment [10].

In humans and animals, E2 is oxidized in the liver, which is quickly oxidized into estrone, which is converted into estriol, is the main product of excretion [11]. The hormone E3 is the main steroid produced during pregnancy and its concentration in the plasma and urine increases constantly during pregnancy [5,8]. Because the hormones EE2, E2 and E3 have low vapor pressure, they are compounds that also have hydrophobicity, thus contributing with the reduction in concentrations of these compounds in the aqueous phase, which may increase their sorption in the soil or sediment [12]. However, oestradiol and ethinylestradiol were found in samples of water supply at concentrations above ng L^{-1} [10].

The presence of E2 (6-66 ng L^{-1}) has also been reported in groundwater near areas with high density of livestock (Peterson et al. 2000). However, contamination of surface water occurs mainly through sewage treatment plants (STPs) [13], once water returns to the supply system without complete removal of contaminants. Besides E2, EE2 also receives special attention because it is continuously excreted into the sewage system, so it is not completely removed at the STPs [8]. Research shows that the estrogen E2 is highly responsible for estrogenic activity in effluents at STPs [14], and it is found in soil and water springs in some countries [14,15] which are mostly used as source of drinking water.

However, decontamination of drinking water will only be attained with the use of new processes for the removal of these compounds [10] such as ozonation, filtration with activated carbon, reverse osmosis, reverse electrodialysis and chlorination. One more affordable techinque is to artificially increase groundwater resources [16]. The use of water contaminated with hormones for human consumption is associated with a decline in sperm counts in men, increased incidence of breast cancer and in the testicles, precocious puberty [17]. Besides, hormones can potentially affect the reproductive system of water

organisms, for example, causing feminization of male fish [5, 18, 19, 20]. Due to low levels of contamination and the complexity of environmental matrices requiring the use of methods with high sensitivity and selectivity for the analysis of strogen residues [1, 16, 21], several analytical methods for hormones determination, using liquid chromatography coupled with several types of detectors have been developed. For detection of residual pharmaceuticals in aquatic environment in the range of ng L^{-1} and µg L^{-1}, the method used in some laboratories are based on solid phase extraction (SPE) and high performance liquid chromatography (HPLC) [22] coupled to a diode array detection [23].

However, in this context, this is the first work dedicated to the determination of hormones in the drinking water supply of the city Piracicaba (Sao Paulo, Brazil). Thus, the main objectives of this paper were (i) analyze and quantify the hormones estriol (E3), 17α-etinilestradiol (EE2) and 17β-estradiol (E2) in samples of raw and treated water from rivers Piracicaba and Corumbataí using high performance liquid chromatography coupled to a diode array detector (HPLC/DAD), (ii) evaluate if the equipment is suitable to monitor contamination in drinking water and, (iv) adapt and validate the analytical methodology for the analysis of hormones in water samples.

2. MATERIALS AND METHODS

2.1. Characterization of the study area

Piracicaba River (Fig. 1) supplies cities that comprise an important industrial and agricultural center and its main contamination is caused by the discharge of wastewater, due to increased population density (over 4 million people), using 95% of the watershed. Another area of study is the Corumbataí River (Fig. 2), which drains into the Piracicaba River and is also used to supply water to a population of 500,000 inhabitants, including the city of Piracicaba, São Paulo, Brazil. Therefore, studying hormone contamination in these basins has become crucial.

Figure 1. Location of collection point on the Piracicaba River in the city of Piracicaba, São Paulo, Brazil (extracted from Google Maps)

Figure 2. Location of collection point on the Corumbataí River in the city of Piracicaba, São Paulo, Brazil (extracted from Google Maps)

2.2. Materials

Solvents utilized were methanol HPLC grade (MTedia Company, USA) and ultrapure water (Pura-Q, Brazil). The analytical standards used were hormone natural estriol (E3; purity 99.0%), hormone synthetic 17α-etinilestradiol (EE2; purity 98.5%) and natural hormone 17β-estradiol (E2; purity 99.0%), both purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and glass fiber filter 0.47 µm (Mackerey-Nagel, Germany).

2.3. Preparation of standard stock solutions

Stock solutions from the standards of E3, E2 and EE2 were obtained from the dilution of 10 mg of standards in 10 mL of methanol and, from the stock solutions, mixed standard of hormones were diluted for the preparation of calibration curve at concentrations of 0.5; 1.0; 2.0; 3.0 and 4.0 ng L^{-1}. The standard solutions were stored in amber glass vials and placed in a freezer (-20ºC) until the time of use.

2.4. Apparatus

Measurements of hormones in water samples were performed by a high chromatograph performance liquid chromatography (Agilent, USA), model 1200 coupled to a diode array detector (DAD), consisting of bomb quaternary, injector manual, degasser, workstation (Agilent ChemStation version B.03.02) for integration data, column Kromasil 100-5C18 (5 µm x 4.6 mm x 250 mm), a guard column Kromasil 100-5C18 and a vacuum chamber Visiprep DL (Supelco, USA). The conditions used were previously optimized and adapted [21].

2.5. Analytical protocols

The operational conditions of the chromatograph were: column maintained at 40ºC, mobile phase constituted by solvents A (methanol) and B (ultrapure water), flow rate of 1.0 mL min^{-1}, manual injection of 20 µL and wavelength 230 nm. The gradient was 0 – 12 min (51% A and 49% B), after 12 – 26 min (49% A and 51% B) and remained under this condition until the end of the chromatographic run.

To determine the efficiency of extraction of solid phase cartridges, cartridge OASIS HLB 500 mg (Waters Corporation, USA) was conditioned with methanol and

ultrapure water (flow 1.5 mL min^{-1}), engaged in a vacuum chamber [15]. While the cartridges were conditioned, 200 ml of sample were filtered through glass fiber filter (0.45 µm), homogenized in ultrasound and added to a cartridge at a rate of 1.5 mL min^{-1}. The cartridges were washed with ultrapure water and dried in a vacuum. The elution was performed with methanol and the eluate was dried in a water bath with N_2 and reconstituted with methanol and water (4 mL) for subsequent HPLC-DAD. For spiked samples, mixed standard was used at concentrations of 1.0, 2.0 and 3.0 ng L^{-1} (containing E3, EE2 and E2).

2.6. Collection of samples

Samples were collected on the surface water, in two points of captures of water at rivers Piracicaba and Corumbataí and treated water at a residence in Piracicaba, São Paulo, Brazil. The samples are collected in triplicate in amber glass bottles of 1 liter each, decontaminated with alkaline soap, running water, deionized water, acetone, solution of 5% of dimethyldichlorosilane in toluene, dichloromethane and river water before sampling. The bottles were totally filled to minimize contact between water and air, then the flasks were transported to the laboratory and stored in a freezer at ± 20°C for later extraction and analysis and the samples were collected monthly from November 2007 to April 2009 (every month), totaling 54 samples in 18 months.

3. RESULTS

Validation of the proposed method was obtained by evaluating the parameters of selectivity, linearity, repeatability and accuracy. The selectivity of the chromatographic method was evaluated by monitoring the absence of peaks in the regions of hormones retention times. Therefore blank samples (ultrapure water) were injected with and without addition of mixed standard.

Linearity was evaluated by the response obtained as a function of analyte concentration. Analytical standards were prepared in solvent and linearity was determined by the correlation coefficient (r^2) obtained from the graph relating the equipment response by means of five analyte concentrations and to estimate the coefficients of standard curves, the method of linear regression was used. After the injections, the relative standard deviation (RSD) or coefficient of variation (CV), the estimated standard deviation (s) and linear regression equation of calibration curve for each hormone were determined (Table 1). The process of recovery assays was used to evaluate the accuracy. Ultrapure water samples were spiked and extracted according to the method in section "Analytical protocols", at 1.0, 2.0 and 3.0 ng L^{-1} and quantified by HPLC-DAD (Table 1).

The linear ranged between 0.5 – 4.0 ng L^{-1} for E2, EE2 and E3. Limits of detection (LOD) and limits of quantification (LOQ) were determined by methodology based on parameters of the analytical curve developed by Shabir (2003) and values are demonstrated in Table 1.

Table 1. Values of equations of straights, correlation coefficient and linear range (in ng L^{-1}), limits of detection (LOD) and quantification (LOQ), both in ng L^{-1}, for hormones E3, E2 and EE2.

Hormone	Linear equation	LOD	LOQ
E3	y = 132.84x – 5.439	0.1	0.5
E2	y = 139.35x – 3.048	0.07	0.5
EE2	y = 124.44x – 1.937	0.05	0.5

Precision was evaluated by estimating the repeatability through calculating the RSD or CV and the estimated standard deviation (s) resulting from the analysis of seven injections of the mixed standard at a concentration of 1.0 µg L^{-1} under the same operating conditions and in the same day (Table 2).

Table 2. Repeatability of the chromatographic method to determine E3, E2 and EE2.

Hormone	Parameter	Rt* (min)	High (mAu)
E3	Mean	9.2	7.14
	Standard Deviation *(s)*	11.94	0.39
	Relative Standard Deviation (RSD)	1.81	5.46
E2	Mean	42.3	1.93
	Standard Deviation *(s)*	8.44	0.21
	Relative Standard Deviation (RSD)	1.09	10.88
EE2	Mean	48.4	2.98
	Standard Deviation *(s)*	10.59	0.24
	Relative Standard Deviation (RSD)	0.78	8.05

*Rt = Retention time.

The method was selective because for the evaluation of blank sample and matrix spiked with E2, EE2 and E3, there was no interference of eluted compounds at retention times of hormones, and they were well separated from other compounds present in the sample. Retention times for the chromatograms achieved for E3, E2 and EE2, were 9.2, 42.3 and 48.4 min, respectively (Fig. 3).

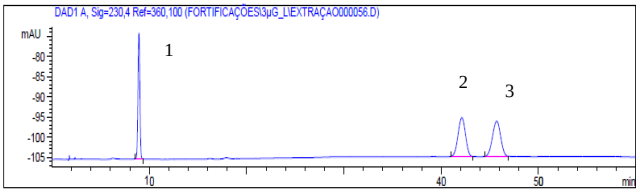

Figure 3. Chromatogram of standards containing estriol (1), 17β-estradiol (2) and 17α-ethinylestradiol (3), analyzed by HPLC-DAD. Retention times for each analyte were (1) 9.2, (2) 42.3 and (3) 48.4 min, respectively.

Linearity of the chromatographic method was achieved by injecting, in triplicate, 5 levels of concentration of mixed standard (containing E3, E2 and EE2) and was observed in the range between 0.5 and 4.0 µg L^{-1} through correlation coefficient (r^2), which were 0.998, 0.998 and 0.999 for E3, E2 and EE2, respectively. The method denoted low dispersion of data, low uncertainty of the estimated regression coefficients and ideal adjustment data for estimated regression line [24].

Estimation of repeatability of chromatographic method was performed to assess the accuracy of the method. Therefore, the estimates for the absolute standard deviation (s) and RSD or CV were calculated from a mixed standard containing hormones E3, E2 and EE2 of known concentration, which was injected seven times under the same operating conditions and in the same day.

The repeatability of this method was evaluated by some parameters like retention time (in minutes) and high of peaks (mAU). The values of intra-assay precision obtained by the mean, standard deviation and relative standard deviation of measures of retention times and peak heights of analytes are described in Table 2. Considering that the desired results for accuracy, evaluated by CV obtained, must be less than 15% [25], the data obtained in the validation of this methodology showed adequate precision, both in the analysis of retention times and in chromatographic peak heights.

Intra-assay precision of the chromatographic peak heights ranged from 5.4% to E3, 10.8% to E2 and up to 8.0% for EE2. The analysis of precision of retention times of the analytes ranged from 1.8, 1.0 and 0.78% for the E3, E2 and EE2, respectively.

4. DISCUSSION

Authors Alda and Barceló (2000) [26], determined the accuracy of the methodology for the determination of hormones by HPLC-DAD and found coefficient of variation greater than 10% for E3, E2 and EE2, which is slightly above the results obtained in this work, using the same detector diode array detector (DAD). Alda and Barceló (2001) [15] obtained CV from 0.9 to 3.4% in an inter-assay precision analyzing the same compounds, in this case, the values are higher than those found in this work, because an on-line system was not used to analyze the samples.

The accuracy of the method was evaluated through recovery experiments and the acceptable ranges of recovery for residue analysis are usually between 70 and 120% accurate to ± 20% [8]. The matrix free from contamination was added to all three hormone concentration standards. The recovery was evaluated by repeating extraction of samples fortified three times at three concentration levels (1.0, 2.0 and 3.0 ng L^{-1}). The data obtained through the injection of the fortifications were used to calculate the standard deviation (*s*) and CV and RSD. The recovery values for E3 ranged from 82.5 to 112.3%, for E2 between 91.4 and 100%, and for EE2 between 93.9 and 126.8%. The values obtained are suitable, within the range of acceptable range of recovery for residue analysis, which is between 70 and 120% accurate to ± 20% [8].

The percentages of recovery of E3 and E2 in the samples of ultrapure water spiked with mixed standard of 3.0 ng L^{-1}, reached a value of 97% and 100% respectively, They were close to those found by Wang et al. (2008) [21], which used the same concentration of fortification in raw water samples of river and obtained values of 91% for E3 and 99% for E2, using cartridge for solid phase extraction (SPE).

Alda and Barceló (2001) [15] obtained recovery percentages from 99, 97 and 97% for hormones E3, E2 and EE2, respectively, using the same type of cartridge, sample volume spiked and concentration of analytes of interest. In the current study, the values of each analyte recovery were very close to those found by [15].

The LOD and LOQ were calculated using the analytical curve for each hormone and there were significant differences between LD values for compounds E3 and the others (EE2 and E2). However, this may be attributed to the fact that this molecule presents chemical bonds that make it less detectable by the diode array detector. The detection limits for the compounds are below the limits founded by Almeida and Nogueira (2006) [23] (50 µg L^{-1} for E2 and 300 µg L^{-1} for EE2) for these two compounds.

Therefore, at the end of validation methodology, the samples were collected and subsequently the method was applied. The raw and treated water samples collected (n = 54) were applied to the methodology adapted and validated and the method did not detect any contamination with the target hormones used in this study (E3, E2 and EE2). It is possible that during the collection period, samples were not contaminated and the contact of water treated with chlorine residual could mitigate potential contaminants in the water, knowing that it degrades these substances [27]. Fig. 4 illustrates the chromatogram of a sample of the Corumbataí River.

5. CONCLUSION

So it is concluded that the chromatographic method SPE and HPLC/DAD proposed for detection and quantification of hormones E3, E2 and EE2 is specific and

linear (concentration range 0.5 to 4.0 ng L^{-1}) and the values of detection and quantification limits were suitable for the analysis of hormones in water samples. This methodology also showed characteristics such as simplicity and reliability, which has potential for application in routine laboratory analysis of hormones E3, E2 and EE2 in aqueous samples. However, no contamination by hormones was detected in 18 samples of raw and 36 samples of treated water from the Piracicaba and Corumbataí Rivers.

ACKNOWLEDGEMENTS

The authors would thanks for support given by CAPES, CNPq and Fapesp.

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