



Journal of
**Pharmacology and
Toxicology**

ISSN 1816-496X



Academic
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Research Article

Steroid Sex Hormone Ethinylestradiol in Recycled Wastewater at Recreational Park

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Abstract

Background and Objective: Birth control pill is one of synthetic steroid hormones from EDCs that are believed to cause higher transgender case and cancer risk in the environment today. The objectives of this research were to (a) identify the concentrations of EDC steroid hormones in recycled wastewater used for drinking purposes and (b) investigate the influence of selective water quality parameters on the occurrence of EDC steroid hormones. **Materials and Methods:** Dispersive Liquid-Liquid Microextraction method was used to extract the steroid hormones and tested using High Performance Liquid Chromatography (HPLC). Results obtained from the samples will be analyzed using Microsoft Excel Office Professional Plus 2013 and Pearson Correlation. **Results:** As a result, the average concentration of EE2 identified was $181.63 \pm 25.12 \text{ ng mL}^{-1}$. From the correlation statistical analysis, dissolved oxygen ($r = -0.975$, $p = 0.0009$) was the most influencing in-situ parameter on EE2 occurrence. **Conclusion:** In conclusion from the result obtained for the recycled wastewater is alarming and worrying as the steroid sex hormone is still presence and detected although in nanogram per milliliter in environment as it will bring and posed a life threat towards the living organism in the environment.

Key words: Endocrine disrupting compounds (EDCs), ethinylestradiol (EE2), wastewater, steroid hormone, drinking water quality

Received: April 29, 2017

Accepted: May 30, 2017

Published: June 15, 2017

Citation: Carolyn Payus, Jessica Roger and Sim Yen Ping, 2017. Steroid sex hormone ethinylestradiol in recycled wastewater at recreational park. J. Pharmacol. Toxicol., 12: 148-153.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The topic on steroid sex hormones from past study¹ and their effects towards the aquatic environment were found to be lacking as compared to other more common water quality parameters studied such as heavy metal contamination, fecal coliform contamination and nutrient contamination. Other than that, there has been no environmental legislation that covers hormones and antibiotics in the context of environmental law up to this date. Hence, this unique group of hazardous organic micro-pollutants is gradually becoming prominent as they could pose a new threat to the decline in water quality with their endocrine-disrupting effects to both human and wildlife, thus this research could propose a new set of provisions pertaining to concentration limits of hormones and antibiotics to combat this emerging threat to the health of aquatic environment¹. The presence of both natural and synthetic steroids, especially in parts per trillion concentrations are worrying because these chemicals are capable of altering the production and activities of endogenous hormones by interacting with the endocrine system and hence causing a potential threat to the human health and aquatic life and now they will affect the drinking water supply since this study has shown that they capable to remain in waste water even after treatment.

Water is crucial for all aspects of life from marine creatures that spend their entire lives in aqueous environment to terrestrial organisms that guard their watery interiors with protective skins the quantity and quality of water both are declining on account of continuous growth of the population, industrialization, food production practices, increased living standards and poor water use strategies². For 5-20% of the total water usage goes to industries which often generate a substantial amount of total wastewater³. Wastewater can be defined as a combination of one or more domestic effluent containing black water such as urine, faecal sludge and grey water which often consists of kitchen and bathing wastewater, water from commercial establishments and institutions which includes hospitals, industrial effluents, storm water and other urban run-off; agricultural, horticultural and aquaculture effluent, either dissolved or as suspended matter⁴.

Endocrine Disrupting Compounds (EDCs) are also found in wastewater. According to Li *et al*⁵, EDC is defined as "any substance or mixture that has the ability to alter the functions or to disrupt the synthesis, secretion, transport, binding, reproduction, development, the behaviour of an organism or its progeny in the endocrine systems and consequently causes

health effects in an intact organism". The EDC steroid hormones include several types of both natural and synthetic hormones such as Estrone (E1), Estradiol (E2), Ethinylestradiol (EE2), Estriol (E3), testosterone and progesterone.

The EDC steroid hormones affect the timing of puberty which is the multifaceted developmental process faced by both males and females⁶. As a result, onset of puberty begins late in childhood and causes a transition period from a non-reproductive to a reproductive state⁷. In addition, studies have shown that EDCs are also responsible for the feminization of fish in the aquatic environment⁸⁻¹⁰. Estrogen also causes endometrial and ovarian cancers and testicular cancer in men. Besides that, these steroid hormones have the potential to decrease sperm counts leading to reproductive disorders in men¹¹.

Wastewater recycling or reclamation is one of the solutions to wastewater management. Wastewater recycling refers to reusing the treated wastewater for beneficial purposes such as industrial processes, toilet flush, agricultural and landscape irrigation. The recycled wastewater for drinking purposes goes through a Reverse Osmosis (RO) treatment to ensure the wastewater can be used as indirect potable water. However, this method does not guarantee a complete removal of the EDCs as treatment processes such as filtration can only remove minor amount of Suspended Solids (SS) that escape from settling in the previous steps¹². Some of the EDC steroid hormones might survive the water treatment and move to the underground water. Conventional Wastewater Treatment Plants (WWTPs) are said to be not designed to remove steroid hormones or their metabolites present in wastewater which thus affects the consumer confidence on the quality of the drinking water^{13,14}.

Steroid hormones as part of EDCs present in drinking water is a human health concern, although safe exposure limits have yet to be established due to the need for further studies to determine the impact of long-term and synergistic exposure¹⁵. The incomplete removal of steroid hormones may cause adverse effects on human beings, aquatic lives and the environment upon consumption. This study advance new knowledge in providing a comprehensive water quality monitoring application on micro-constituents pollutants for the non-traditional human waste contamination markers mainly on steroid hormones. Other than that, there has been no environmental legislation that covers steroid hormones up to this date. Hence, this unique group of hazardous organic micro-pollutants is gradually becoming prominent as they could pose a new threat to the decline of water resource quality. The research is significant to develop a database and

standard or allowable limits for the non-traditional contaminants. Thus, this research aims to (i) identify the concentration of EDC steroid hormone in the recycled wastewater used for drinking purposes and (ii) investigate the influence of selected water quality parameters on the occurrence of EDC steroid hormone.

MATERIALS AND METHODS

Study area: This study was carried out from 24th September, 2016-11th March, 2017. Recreational Park P studied is not only famous for its musical fountain but also for its jogging trail. It is located at latitude 5°56'58.92" and longitude 116°2'54.96". The park has its own wastewater treatment plant using reverse osmosis system designed by a Quabella (Model:ROC 4313). Upon treated and recycled, the water is channeled to water dispenser for public drinking use, thus this does not ensure the complete removal of the steroid hormones from the recycled wastewater. Thereby, for the sampling points, the recycled wastewater was taken from the water dispenser provided.

Materials and apparatus: Deionized water purification in Mili-Q water purification system, dimethyl sulfoxide by Merck (DMSO>99.99%), HPLC Grade Acetonitrile, Methanol by Merck, 1-Undecanol, Nitric acid by Lab-Scan, Ethinylestradiol (>99%) by Sigma Aldrich, centrifuge tubes, 0.45 µm membrane filters, auto sampler vials, scoop, High Performance Liquid Chromatography (HPLC), mercury thermometer, Meter Toledo pH meter and multi-parameter (YSI) meter.

Procedures: Standard stock solution of Ethinylestradiol (EE2) $C_{20}H_{24}O_2$ was prepared at a concentration of 2000 mg L⁻¹ in acetonitrile. Standard solutions were diluted to concentrations of 50, 550 and 1550 ng mL⁻¹ to obtained a linear calibration graph ($R^2>0.99$) as shown in Fig. 1. Working standard solution was prepared at a concentration of 200 ng mL⁻¹. All solutions were stored and maintained at 4°C until ready to use.

Test for steroid hormones: Ten mL water sample was filtered, filled into a centrifuge tube and spiked with 50 µL 1-undecanol (extraction solvent) and 200 µL methanol (dispersive solvent). A cloudy appearance will form and the solution was immediately centrifuged at 4500 rpm for 3 min. Then, immediately place the tube in an ice bath for 10-15 min. Solid organic droplets formed will be extracted and transferred into the auto sampler vial. At room temperature, the solid organic drop will melt very quickly in the vial. 35 µL of DMSO will be added into the vial and then sent for HPLC analysis^{16,17}.

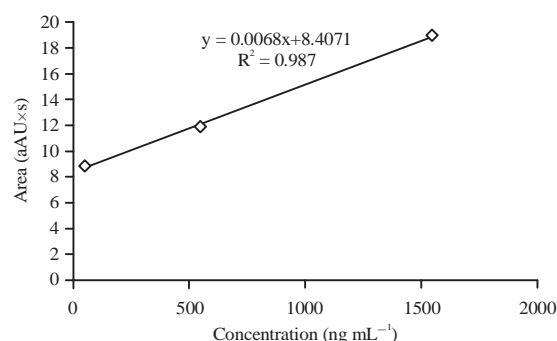


Fig. 1: Calibration curve of Ethinylestradiol (EE2)

Validation of method for HPLC system: The developed method was evaluated by using various parameters such as linearity, correlation coefficient, Limit Of Detection (LOD) and Limit Of Quantification (LOQ). All relevant data were input into an excel sheet. The calibration curve was drafted out to find the linearity equation and correlation coefficient (r). The calculations are shown below¹⁵:

$$\text{SD of intercept} = \text{SE} \times \text{No. of samples} \quad (1)$$

$$\text{LOD} = \frac{(\text{SD of intercept})}{\text{Slope}} \times 3.3 \quad (2)$$

$$\text{LOQ} = \frac{(\text{SD of intercept})}{\text{Slope}} \times 10 \quad (3)$$

Statistical analysis: Results obtained from the samples will be analyzed using Microsoft Excel Office Professional Plus 2013 and Pearson Correlation. Statistical analysis by Pearson Correlation was used to determine the relationship between the in-situ parameters with the steroid sex hormone concentrations and their presence with coefficient value, $r \geq 1$. The p value was also determined to show if there was a significant value or not with either ($p < 0.05$, $p < 0.01$)¹⁸.

RESULTS AND DISCUSSION

From the findings obtained for the recycled wastewater used as drinking water in this recreational park is alarming and worrying as the steroid sex hormone is detected still presence although after the treatment. Although the recreational park has its own wastewater treatment plant however, in this research it does not ensure a 100% complete removal of the steroid hormones from the recycled wastewater and it is more worrying as the recycled water are supplied to the park user for free through water dispenser including children. The

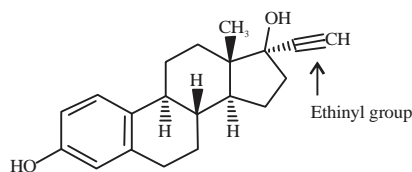


Fig. 2: Chemical structure of 17 α -ethynylestradiol¹⁹

conventional Wastewater Treatment Plants (WWTPs) are believe not designed to remove organic micro-pollutant such as the steroid hormones or their metabolites in wastewater which thus affects the consumer on the quality of the drinking water^{3,4}.

In this research, before the sample analysis was done it was very important to do the data validation in order to obtain more precise results especially dealing with lower value of concentrations such as steroid hormones compound. Thus, in order to obtain that, calibration curve for the Ethynylestradiol (EE2), the Limit Of Detection (LOD), Limit Of Quantification (LOQ) and accuracy were calculated and stated as in Fig. 1.

The retention time of EE2 ranged from 2.587-2.616 min whereby the average of retention time was about 2.601 ± 0.005 min. The Limit Of Detection (LOD) and Limit Of Quantification (LOQ) for this method was 195.010 and 590.931 ng mL⁻¹, respectively. Sampling was carried out on weekends and weekdays to determine if there would be a difference in concentration of EE2 detected in the recycled wastewater used as drinking water due to the population of people visiting during a weekday and a weekend. Based on the result obtained it is found that there were often detections and occurrences of steroid sex hormone in recycled wastewater in the recreational park P. The highest concentration detected was on weekdays with average reading of 280.32 ± 46.80 ng mL⁻¹ whereas the lowest concentration detected was on weekends with average reading of 82.94 ± 47.22 ng mL⁻¹. The concentration of EE2 detected on Weekends was lower compare to Weekdays as some of the EE2 may have been adsorbed to sewage sludge and removed from the water during wastewater treatment processes¹⁷. EE2 is believed excreted in the urine and feces hence probably due to the inefficiency in the treatment system; some of the EE2 entered the recycled wastewater used for drinking purposes. In addition, the main reason for the detection of EE2 in the recycled wastewater is its chemical structure.

Among the estrogens, EE2 is the most difficult to degrade and said to be most potent^{19,20}. It has an ethinyl group in the position of 17 α which shields the compound from oxidation²¹,

hence becoming a stable compound which is capable of entering the environment and thus the recycled wastewater. According to Mazellier *et al.*²², ethynylestradiol is said to be more recalcitrant in the water body because it has an ethinyl group at C-17 atom as shown in Fig. 2. This feature prevents the oxidation of this C-atom, hence, ethynylestradiol has greater influence on the environment for the reason of its stability in the water body^{23,24}. Unlike synthetic estrogen, natural female sex hormone is not as potent and persistent compare to synthetic. EE2 has high resistance to degradation and greater oral bioavailability in the human body is one feature that makes it useful in contraceptives¹⁰. According to the physicochemical properties of the synthetic estrogens, it can be said that ethynylestradiol is a type of non-polar and hydrophobic organic compound with low volatility and is more biodegradation resistance^{11,20}. This can explain why the synthetic shows more occurrences and existence compare to natural sex hormone. Ethinyl group which is triple bond attached at C-17 atom and the strength of bond in a chemical structure increases with the increase number of bond. Single bond < double bond < triple bond as shown in Fig. 2 taken from¹⁹.

From the statistical analysis, the relationship between pH, temperature and Dissolved Oxygen (DO) with EE2 was carried out. The correlation coefficient (r) was -0.688 (p<0.05), -0.389 (p>0.05) and -0.975 (p<0.05) for pH, temperature and dissolved oxygen, respectively. All three parameters showed a negative relationship with EE2; depicting that when one of the parameter reading increases, the concentration of EE2 detected will decrease and vice versa. The increased DO levels fasten the degradation rate of EE2 due to the reactive phenolic group thus resulting in a decline in EE2 concentration in the water. EE2 to has a 290-350nm light absorption band region ion which directly induces photochemical decomposition. According to Delclos *et al.*⁹, increasing levels of dissolved oxygen fastens the degradation rate of EE2, hence decreasing the concentration of EE2 in the water. The degradation of EE2 was mainly caused due to the oxidation of photogenerated Reactive Species (RS) and the contribution of direct photodegradation to EE2 degradation was lower than 27%. Therefore, as the dissolved oxygen content increases, there will be an increase in the yields of excited EE2 and the Reactive Species (RS). This reactive species will react with the EE2, causing it to degrade.

EE2 is a synthetic compound that exhibits strong acidity with pKa = 10.33. The degradation of EE2 may potentially be occurring as the high pH (pH> 4.0) of water catalyzes the degradation rate of EE2 in water²⁵. When the water pH > 4.0, it can initialize the oxidation process such as ozonation to

increase the efficiency of EE2 being removed. The increased temperature triggered gonadal growth and maturation in both female and male. This occurs as EE2 overrides its effects and thus, disrupting the terminal step of steroidogenesis where steroids generated. Concentration of EE2, ng mL⁻¹ in cholesterol group will be converted into other types of steroids. Therefore, the reproduction system of living organism will be affected by the effects of temperature on the EE2 presented in Routledge *et al.*²⁶. Theoretically, one of the effects of temperature on ethinylestradiol is that it will suppress the gonadal growth in males that will cause more male infertility and endocrine disruptions.

CONCLUSION

It is concluded that in a nutshell, there was a detection of steroid sex hormone Ethinylestradiol (EE2) in the recycled wastewater used for drinking purposes in the recreational park. The results obtained were higher than the readings obtained by past researchers thus indicating a strong need to monitor and control the waste water treatment plant. It is crucial to recheck and improve the treatment plant effectiveness so as to be capable of removing the EDC steroid hormone. Risk assessment can also be carried out for future study to conduct evaluation and defining the potential impacts and consequences of EE2 occurrence in our environment. This research is beneficial in protecting the public from the impacts of the EDC steroid hormone especially in drinking water resource.

SIGNIFICANCE STATEMENTS

This study discovers the possible existence of steroid hormone residue in recycled wastewater, that is used as drinking water. The data from this study is also beneficial for developing database and standard or the allowable limits for the non-traditional contaminants of steroid hormone. This study will help the researcher to uncover the critical areas of water pollution especially in recycled wastewater drinking water quality that many researchers were not able to explore.

ACKNOWLEDGEMENTS

This study is financially supported by Ministry of Higher Education, Malaysia (MOHE) through Fundamental Research Grant (FRG0456), another grant from Niche Research Grant Scheme (NRGS0005) and Universiti Malaysia Sabah (UMS) Grant through (SBK0237).

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