METHOD VALIDATION FOR 17α- ETHYNYLESTRADIOL (EE2) DETECTION USING HPLC

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ABSTRACT

Although the presence of estrogenic compounds in water bodies may not be significant in amount, but its impact on the ecological system is significant enough to draw attention of researchers. Major effects of estrogenic pollutions to the aquatic organisms include change in female gonadal phenotype, decrease in fertility, and fish feminization, that, eventually leads to depopulation. Among the major sources of estrogenic pollution are contraceptive pills, hormone treatments and discharges of humans and animals which usually end up in sewage treatment plants. As vital as it is to investigate the presence of estrogenic pollution in water bodies, there is a lack of simple validated method to determine the level of estrogenic pollution in water bodies. Currently, gas chromatography with high-resolution (HRGC/HRMS) is the only standard procedure for hormone identification under the Clean Water Act. However, this method is not widely applied due to the non-availability of HRGS/HRMS in most common laboratories. Thus, this current research investigates and validates the suitability of a rapid and accurate estimation of 17α-Ethynylestradiol (EE2) using a high performance liquid chromatography (HPLC), an equipment commonly available in most research laboratories. This method validation is done in accordance with the guideline used for pharmaceutical drug detection, which include system suitability, system sensitivity, system linearity, accuracy and precision. The suitable wavelength of the HPLC was detected at 280 nm, while, the limit of detection and quantification, each recorded at the differential height of Δ 0.0465 mAU and Δ 0.1550 mAU. The accuracy and precision of the system were validated at coefficient of variance in the range of 0.01% to 0.09%, which is much lower than the accepted value of 5%. Lastly, the validation of system linearity gives a regression value of 0.9993. Thus, this method is deemed valid for the detection of EE2 in aqueous solution.

Keywords: 17α-Ethynylestradiol, estrogenic pollutions, high-performance liquid chromatography, method validation.

INTRODUCTION

The presence of estrogenic compounds is water bodies has been reported to pose a significant impact to the aquatic life. Estrogens in the environment cause the adaptation of aquatic organisms to the exposure by modifying their characteristics, causing female gonadal phenotype, decrease in fertility, and fish feminization [1-6].

Sources of these estrogenic compounds are not limited only to contraceptive pills and growth promoter but also discharge of human and animals that end up in sewage treatment plants [6-8]. Thus, wastewater treatment plants have become the cumulative center for estrogenic compounds which are subsequently released into water bodies after treatment [9].

Methods that have been employed to determine the concentration of estrogens so far are HPLC-MS, GC-MS and vitro bioassay. However, all these researchers used a unique method of detection on their own. According to the EPA standard, high-resolution GC combined with high-resolution mass spectrometry (HRGC/HRMS) are required for hormone identification under the Clean Water Act (CWA) (U.S. Environmental Protection Agency, 2007). This standard, however, is not employed by most researchers due to its unavailability in most research labs. Whereas, for HPLC, the standard method for the determination of EE2 is yet to be recorded.

In this research, HPLC and C-18 column that are commonly available in any general laboratory, were used. This research validates and determine the suitability of a rapid and accurate estimation of EE2 through a validated method of HPLC.

METHODOLOGY

A stock solution of 1000 µg/mL was prepared from 98% purity EE2 from Sigma Aldrich where 0.5 g of EE2 is weighted in an analytical balance before being mixed in an amber laboratory bottle with 500 mL of HPLC grade methanol. For each required concentration, the volume required is calculated based on $M_1V_1=M_2V_2$ equation where M is the concentration of EE2 and V is the volume required.

Whereas, standard solution was prepared by serial dilution producing standard concentration at 50 µg/mL, 10 µg/mL, 1 µg/mL, 0.5 µg/mL and 0.1 µg/mL for linearity test. Each replicate for standard solution was prepared from the initial EE2 from Sigma Aldrich to minimize human error during the dilution process.

HPLC 1260 Infinity Series with Column Zobrax SB-C18 by Agilent Technologies was used for the whole research project. Column Zobrax SB-C18 was used together with acetonitrile and deionized water at a ratio of 45:55. The flow rate of the HPLC instrument was adjusted to 1 mL/min, a temperature of 30 °C, and injection volume of 90 µL were used. These conditions selected were modified from Sigma Aldrich application report for Waters system [10].

To ensure an accurate quantitation in the research, a series of method validation procedure must be conducted. Among the requirement considered for the validation method are system accuracy, precision, detection and quantitation limit, linearity and range [11].

For determination of system suitability at different wavelengths, two concentrations representing a low concentration at 2 µg/mL and a high concentration of 100 µg/mL were applied to the HPLC. The analysis was subjected to series of wavelength ranging from 200 nm to 340 nm. Each experimental run was repeated 6 times. The system suitability was then assessed by obtaining the percentage of the relative standard deviation (%RSD).

The system sensitivity was determined by the limit of detection (LOD) and limit of quantitation (LOQ) [11, 12]. The detection limit is the lowest amount of analyte that can be detected, but not necessarily be quantified. Whereas, quantification limit is the lowest amount of analyte that are being quantified. An approach based on a signal-to-noise ratio is suitable for systems that shows baseline noise and the limit of detection is generally acceptable when the signal-to-noise is between 3 or 2:1 [11, 12] and thus, evaluation by signal to noise ratio is employed in this validation. Signal to noise ratio define the lowest concentration detectable in a peak area of three times the baseline noise for LOD and the lowest concentration detectable in a peak area of ten times the baseline noise for LOQ. The lowest concentration in the study range of 0.1 µg/mL is used to gauge the system sensitivity.

The accuracy and precision of this method are evaluated by determining the intra-day variations which represent the repeatability of experiments and inter-day variations which is the intermediate precision where a difference of 3-day evaluation was done. To ensure the consistency of the methods, a total of 6 replicates is done on each variation each at 3 concentrations. The concentrations used are 0.5 µg/mL, 5 µg/mL and 10 µg/mL. Evaluation of the system accuracy and precision were done based on the calculated standard deviation and coefficient of variance.

Finally, system linearity or calibration curve is a procedure that runs on a range of known concentration to obtain an unknown result direct proportion to the linearity. The linearity of this system is evaluated by running a set of concentration of EE2 at 0.1µg/mL, 0.5 µg/mL, 1.0 µg/mL, 10.0 µg/mL, 50.0 µg/mL and 100µg/mL at the total of 6 points. These calibrations were done in triplicate where the average value is used to plot the curve. The degree of linearity of the plotted graph is obtained by evaluating its regression line. The system linearity is acceptable when the regression line obtained has a value of more than 0.95.

RESULTS AND DISCUSSION

Determination of system suitability at different wavelengths

The maximum adsorbent wavelength of EE2 is studied at the range of 200 to 340 nm. The retention time is found to be 12.53±0.2 min for overall retention time ranging from low concentration of 2 µg/mL to high concentration of 100 µg/mL to ensure the validity of the reading obtained. Figure 1 shows a comparison graph of absorbance of EE2 at different wavelength. Although wavelength at 200 nm gives a better reading, it is not a suitable wavelength to be used as the UV cut-off point for acetonitrile and deionized water is at 190 nm, and a wavelength of 200 nm is considered too close to this value as seen in Figure 1. Apart from wavelength 200 nm, wavelength 280 nm is the only wavelength that gives a smooth and complete peak.

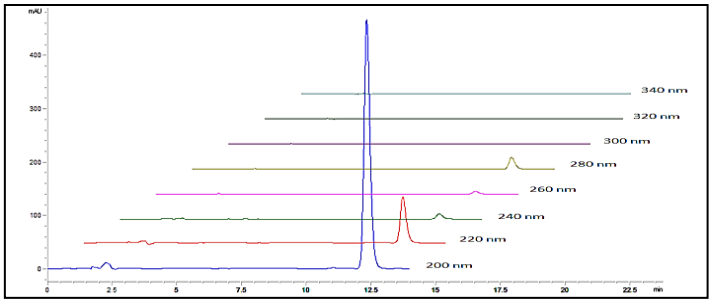

Figure 1: Absorbance of EE2 at a range of wavelength from 200 nm to 340 nm.

Furthermore, the % RSD obtained at a wavelength of 200 nm for low concentration is 3.61%, while 220 nm is 6.00% in contrast to 1.43% obtained at a wavelength of 280 nm from 6 replicates as shown in Table 1. Although all three wavelength gives a %RSD of less than 0.5% at high concentration, % RSD for 200 nm and 220 nm are unable to fulfil the method suitability of less than 2% at low concentration [12] as in Table 1. Thus, a wavelength of 280 nm is selected to be used throughout the EE2 analysis.

Table 1: Relative standard deviation for n=6.

Concentration 2 µg/mL (Low Concentration)			
Wavelength	200	220	280
Replicate 1	2.32	2.35	2.21
Replicate 2	2.15	2.11	2.28
Replicate 3	2.39	2.49	2.28
Replicate 4	2.34	2.49	2.28

Replicate 5	2.26	2.26	2.21
Replicate 6	2.21	2.21	2.21
Mean	2.28	2.32	2.24
%RSD	3.61	6.00	1.43
Concentration 100 µg/mL (High Concentration)			
Wavelength (nm)	200	220	280
Replicate 1	112.45	112.59	112.11
Replicate 2	112.12	111.93	112.36
Replicate 3	111.76	111.61	112.08
Replicate 4	111.54	111.50	111.76
Replicate 5	111.39	111.45	111.27
Replicate 6	111.39	111.45	111.69
Mean	111.78	111.75	111.88
%RSD	0.35	0.36	0.31

System sensitivity: limit of detection and quantitation

The differential height obtained for the baseline noise is Δ 0.0155 mAU. For LOD, the signal to noise ratio is set to 3:1, which is three folds the height of baseline noise where any reading recorded with the differential height of Δ 0.0465 mAU or above is acceptable. Whereas for LOQ, ratio of 10:1 which is ten folds the height of the baseline noise is employed. The acceptable differential height for LOD and LOQ can be seen in Table 2.

Table 2: LOD and LOQ for the system based on signal to noise ratio.

	Signal to Noise Ratio	Accepted height
Baseline Noise		Δ 0.0155 mAU
LOD	3:1	Δ 0.0465 mAU
LOQ	10:1	Δ 0.1550 mAU

Whereas, for further assurance of the system sensitivity and the validity of data recorded, Figure 2 indicates a 0.1 µg/mL concentration of EE2 which is the lowest point of the calibration curve. The height obtained from the graph is 0.17 mAU which is still considered as a higher reading compared to the limit of quantitation which requires only Δ 0.1550 mAU in height. Comparing the lowest possible reading in the experimental runs and both the accepted height for the LOD which is Δ 0.0465 mAU and LOQ which is Δ 0.1550 mAU, all the recorded data in this research is deem valid and in the acceptable range that is required in this method validation.

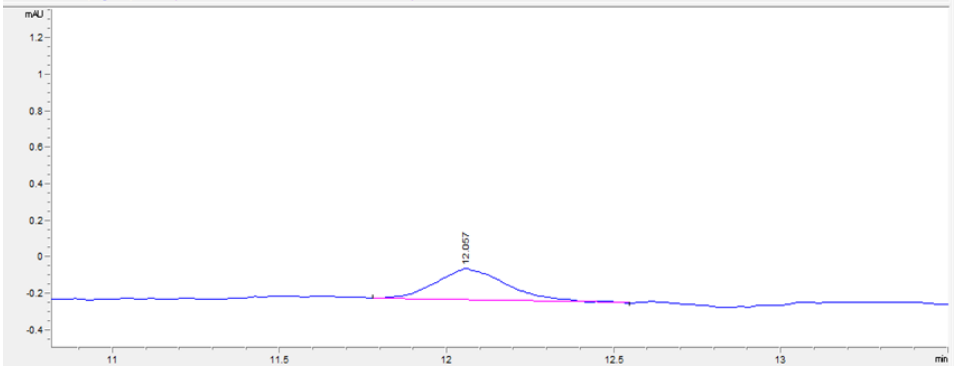

Figure 2: Concentration of EE2 at 0.1 µg/mL.

Accuracy and precision

The accuracy and precision of the method are obtained from the intra and inter-day study as given in Table 3. The intra-day readings show standard deviations in the range of 0.05 to 0.16. The precision of the method is determined by the coefficient of variance reading, which are in the range of 0.01% to 0.09%. A good precision is also obtained from the intermediate precision which is the inter-day precision value. Since all the coefficient of variance obtained from the intra-day and inter-day variations are much lower value than the accepted value of 5%. Therefore, this method is considered to be meeting the requirement for system accuracy and precision testing.

Table 3: Intra and inter-day accuracy and precision study.

Day	Concentrations (µg/mL)	Mean (µg/mL)	Standard Deviation	Coefficient of Variance (%)
1	0.5	0.52	0.05	0.09
	5.0	5.44	0.16	0.03
	10.0	11.04	0.12	0.01
2	0.5	0.54	0.02	0.03
	5.0	5.47	0.11	0.02
	10.0	11.02	0.12	0.01
3	0.5	0.53	0.01	0.03
	5.0	5.41	0.13	0.02
	10.0	11.00	0.13	0.01

Detection linearity (calibration curve)

A calibration curve is plotted for this experimental run based on the concentration of EE2 within the range of 0.1 to 100 µg/mL, with a total of 6 points. The detection wavelength is fixed at 280 nm. The regression coefficient, R^2, obtained from this standard curve is 0.9993 and the slope is found to be 3.226 as shown in Figure 4. This gives the regression equation at y=3.226x. This system linearly is valid as the R^2 obtained is above the value of 0.95. Thus, the calibration curve is deemed suitable to be used throughout the whole research for data collection.

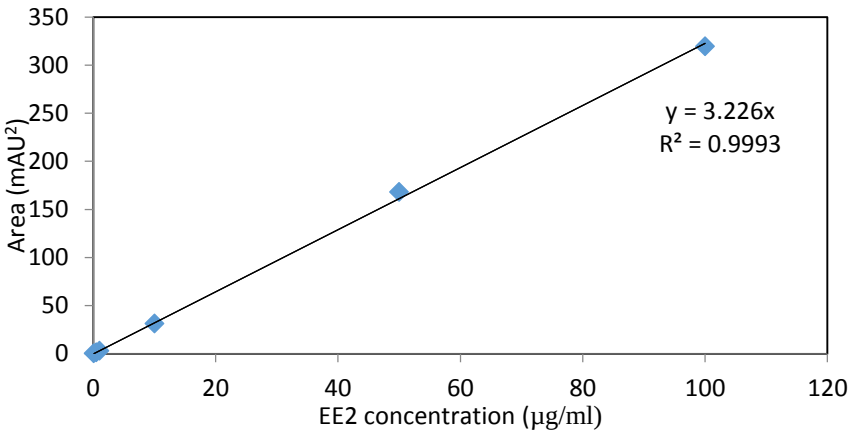

Figure 4: Calibration curve for the validation of linearity.

CONCLUSIONS

An EE2 detection method is validated using HPLC for the range of EE2 from 0.1 µg/mL to 100 µg/mL. A suitable wavelength was identified at 280 nm with the retention time at 12.53±0.2min. Based on the limit of detection and limit of quantification of the system, all data recorded is valid and it is bound within the range. The accuracy and precision of the method study with the EE2 compound are found to be acceptable given the coefficient of variance are all below 5%. Lastly, the detection of linearity gives a standard curve of 0.9993 regression value, validating the equation found at y=3.226x where y detection area in HPLC and x is the unknown EE2 concentration.

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