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Short Communication

Comparing Methods of 17α-ethinylestradiol (EE2) Determination in Surface Water

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Abstract

We compared methods of determination of 17α-ethinyloestradiol (EE2) in surface water in Kielce, Poland. EE2 plays a role as a contraception agent and it is commonly used as an active agent in contraception pills. During studies three methods of EE2 determination were checked: TLC, HPLC, and ELISA assay. Samples of contaminated water were taken from eight points on four rivers crossing Kielce city, tap water, and sludge in a sewage treatment plant (STP). Assays revealed that EE2 is present in river water in amounts of 3.2 ng/L to 6.29 ng/L. The EE2 removal process in STP is ineffective, removings only 1.3%. EE2 was not detected in Kielce's tap water. No correlation between fecal microbial contamination and EDS presence was determined.

Keywords: endocrine disrupting substances (EDS), ELISA, HPLC, 17α-ethinyloestradiol (EE2), microbiology

Introduction

Endocrine disrupting substances (EDS) are believed to disrupt regular endocrine functions after entering the bodies of humans or animals. EDS operate like hormones and are novelly treated. The earliest report on human hormones in water was published in 1965, indicating that steroids were not completely eliminated during wastewater treatment [1]. EDS like 17α-ethinylestradiol (EE2) are in common use as an active ingredient in oral contraceptive pills. Human excretion of estrogens is thought to be the principal source of this type of compound in an aquatic environment and has been estimated at 2.7 mg/L in urine per capita on a daily basis [2]. Estrones were detected in 2/3 of the surface water samples, but in only 1/5 of the groundwater samples in Austria [3]. Unmetabolised EE2 is excreted with urine to sewage and doesn't decompose in sewage treatment plants (STP). SPT efficiency in EE2 removal are up to 78% [4], and it results in presence of EDS in surface waters. Sorption EDS on humic acids was observed. A drop in EDS concentrations in the presence of humic acids was up to 51.6% [5]. Steroid concentrations in raw sewage in German and Catalonian plants ranged from less than 2.5 to 115 ng/L for estrone (E1), less than 5.0 to 30 ng/L for 17β-estradiol (E2), and less than 5.0 to 10 ng/L for 17α-ethinylestradiol (EE2). Estriol (E3) varied from less than 0.25 to 70 ng/L [6]. Methods commonly applied for steroid concentrations are SPE [5], and for determination are GC-MS, LC-MS/MS [5, 6], LC-DAD [7], ELISA [8, 9], and TLC [10]. The presence of EE2 in surface water for sure may affect human maturation processes or fish growth. Recent studies have demonstrated that estrogens at a concentration of 5-6 ng/L are dangerous for populations of fish in lakes [2]. The compounds have a wide range of chemical structures, but all of them have the capacity to disrupt normal hormonal actions. The aims of presented studies were a comparison of three methods of EE2 determination in surface water of Kielce city.

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Materials and Methods

Samples were collected in sterile 1L flasks from March to October 2009, at eight points on four rivers in Kielce city. Samples from raw sewage, STP-treated sewage, and tap water were collected in 2010. For determination of levels of EDS in surface water three methods were used.

Solid-Phase Extraction (SPE)

Concentrations of the tested samples were carried out using solid phase extraction. The solution was concentrated on small columns Oasis HLB 30 mg conditioned with 4 ml of methanol and 4 ml of water. After concentrating the samples, columns were washed with 1 ml of water and eluted with 0.5 ml hexane and 0.5 ml of methanol. The sample was then evaporated and dissolved in 1 ml of acetonitrile.

Thin Layer Chromatography (TLC)

Separation was performed on AL SIL G 250 μm Whatman plates. A mixture of ethyl acetate:chloroform (2:8 v/v) was used as a solvent. Sample separation lasted 180 min. Plates were sprayed with a 50% phosphoric acid that visualizes steroids as "grease spots" [11].

High-Performance Liquid Chromatography (HPLC)

Measurement was performed on a Lachrom device (Merck, Hitachi) with UV L-7400 detector and column LiChrospher 100 RP-18 5 mm (Merck).

Enzyme-Linked Immunosorbent Assay (ELISA)

Determination of amounts of EE2 was performed according to kit producer protocol (Enviro Chemicals, Ltd. Japan), absorbance was measured at 450 nm on an EL340 Plate Reader (Biotek). Concentrated samples were dried in a nitrogen stream and dissolved in 10% methanol.

Microbiology Assay

Total bacterial count in water was measured according to PN EN-ISO 6222:2004 norm [12]. Membrane Filtration was used for coliform determination. Membrane filters (sterile, cellulose acetate, 47 mm diameter and pore size 0.2 μm, Waters) were used with a Sartorius funnel. Determination was performed in 100 ml of raw water according to norm: PN-EN ISO 9308-1:2004 [13].

Results and Discussion

TLC method for EE2 revealed detection limit on 6.25 μg per dot. Samples migrated in an expected way and resulted in Retention Factor R_f=0.69. The amount of EE2 detected by TLC was hundreds of times higher than that recorded in environmental samples [6]. The more sensitive methods needed for environmental assays were applied. By HPLC method the level of EE2 was measured in Kielce surface water from 3.1 ng/L to 50 ng/L, depending on the sample. The HPLC method was sensitive enough for detection of EE2 in environmental samples. However, results differ for the same sample so the method used was not reliable.

Fig. 1. Map of Kielce with marked sampling sites: 1 and 3 – Silnica River, 4 and 5 – Sufraganiec River, 6 – Lubrzanka River, 7 and 8 – Bobrza River. 2. Reservoir Kielce on Silnica River, (http://www.gis.kielce.eu/geoportal_toolkit/map.php).

Table 1. Amounts of 17α-ethinylestradiol (EE2) measured by HPLC method in Kielce surface water.

Sampling site	Month (EE2 in ng/L)	
	III	IV
1	3.1	5.6
2	-	7.3
3	-	42.3
4	50	29.5
5	-	4.8
6	-	8.1
7	-	8.7
8	-	8.7

-not detected

In the next step, EE2 ELISA assay was performed according to manufacturer protocol. Assay was performed in triplicate and standard deviation was calculated. The water samples were collected on the same sampling sites in time presented in Table 2. Assay revealed the amount of EDS in samples in range from 3.2 ng/L to 6.47 ng/L.

In order to detect the efficiency of removal of EE2 by STP, the amount of EE2 was determined by ELISA method. In raw sewage in STP, Kielce was 3.87 ng/L. After treatment process the EE2 levels decreased only to 3.82 ng/L. No significant reduction was observed. Since the sources of EE2 are contaminated water, the total amount of bacteria as well as fecal contamination of surface water was determined. Microbiology assays showed bacterial contamination in all samples of water, tested by colony forming units on agar and ENDO plates.

The detected amount of psychrophilic (22ºC) and mesophilic (36ºC) bacteria revealed that tested water belongs to the first class in order of microbiology contamination. No correlation between EDS presence of amount and bacterial contamination was found.

Conclusions

Performed assays showed that endocrine disrupting substances (EDS) are present in surface water in Kielce city. ELISA method seems to be most reliable and efficient method for detection of EE2 in surface water. Other studies reveal more sensitive methods than those used in this paper: Zhang and Zuo present results gained with GC-MS chromatography with new derivatization methods, where detection limit for EE2 was 0.05 ng/L [14, 15]. The limitation of GC-MS method is the derivatization stage, where investigated estrogens can be partially converted to other estrogen derivatives in organic solvents and their concentrations could be over-estimated [16]. Measured amounts of EE2 in natural water bodies in Kielce city varied from 3.2 ng/L to 6.28 ng/L. The detected amount of EE2 in Kielce surface water was comparable with contamination of Paris, Rome, and Spain [4]. STP does not remove detected amounts of EE2 microcontamination from water.

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Table 2. Amounts of 17α-ethinylestradiol (EE2) measured by ELISA method in Kielce surface water.

Sampling site	Month (EE2 in ng/L)													
	V	δ	VI	δ	VII	δ	VIII	δ	IX	δ	X	δ	XI	δ
1	4.35	0.64	6.27	0.27	6.08	0.03	6.47	0	6.4	0.05	5.76	0.35	5.83	0.01
2	6.36	0.38	3.2	0.19	5.82	0.11	4.51	0.73	6.29	0.05	5.27	0.33	6.29	0.01
3	6.27	0.29	5.26	0.16	5.64	0.39	5.67	0.1	6.32	0.03	4.1	0.33	5.23	0
4	5.99	0.34	6.18	0.23	6.32	0.51	6.12	0.07	6.08	0.05	4.1	0.36	5.36	0.02
5	5.94	0.55	6.11	0.4	5.9	0.36	6.45	0.02	6.03	0.01	4.6	1.4	4.74	0.28
6	5.54	0.18	5.76	0.12	6.28	0.04	5.53	0.24	5.6	0.01	4.78	2.09	5.69	0.08
7	5.06	0.26	5.93	0.21	5.9	0.14	5.75	0.15	6.23	0.1	4.2	2.4	5.93	0.05
8	6.34	0.47	3.98	0.25	5.26	0.08	5.93	0.11	6.08	0.03	4.09	2.51	5.31	0.03

Table 3. Total bacterial count (CFU/ml) from March to October in eight sampling sites.

Sampling site	Incubation temp.	Month (CFU/ml)								
		III	IV	V	VI	VII	VIII	IX	X	XI
1	22ºC	356	488	654	559	709	811	823	656	698
	36ºC	265	280	321	343	446	391	457	333	405
2	22ºC	468	564	892	974	910	845	1031	879	986
	36ºC	257	365	468	591	603	436	257	465	323
3	22ºC	856	2048	1874	1962	1564	1980	1798	1911	1654
	36ºC	616	484	874	653	441	561	387	465	579
4	22ºC	498	515	463	629	587	564	550	519	467
	36ºC	298	317	369	387	320	364	398	277	306
5	22ºC	568	698	717	653	644	690	571	546	641
	36ºC	216	496	514	526	554	501	532	428	407
6	22ºC	217	359	302	416	318	356	299	311	359
	36ºC	220	245	215	283	198	181	265	212	235
7	22ºC	1160	1648	1542	1795	1123	990	1298	1987	1327
	36ºC	1176	528	633	894	872	714	645	688	547
8	22ºC	2060	2248	2350	2477	2130	2589	1890	1798	1320
	36ºC	648	644	699	546	635	733	924	645	501

Table 4. Membrane filtration results in ENDO agar plates from March to October in eight sampling sites (CFU/100 ml).

Sampling site	Month (CFU/100ml)								
	III	IV	V	VI	VII	VIII	IX	X	XI
1	2	1	9	13	11	*	*	1	0
2	0	0	1	4	8	0	3	1	6
3	0	2	1	4	0	1	0	0	0
4	1	3	1	15	0	1	9	0	0
5	0	7	9	11	0	4	6	0	0
6	0	0	0	0	0	0	0	0	0
7	0	1	0	2	4	0	0	1	0
8	2	0	0	5	1	1	3	0	0

* uncountable

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