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Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands

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Abstract

An analytical procedure was developed that enables routine analysis of four estrogenic hormones in concentrations below 1 ng/l in surface water and waste water. The recovery was 88–98% with a limit of detection of 0.1–2.4 ng/l depending on the compound and the matrix measured. This method was used to determine the occurrence of 17β-estradiol, 17α-estradiol, estrone and 17α-ethinylestradiol in the aquatic environment in The Netherlands. The data show that estrogenic hormones can be detected at low concentrations (up to 6 ng/l) at some locations in surface water. In selected effluents of waste water treatment plants estrone and 17β-estradiol were detected in concentrations in the ng/l range. Concentrations of 17α-estradiol and the contraceptive 17α-ethinylestradiol were in most of these samples below the limit of detection. Hormone glucuronides were not detected in most surface water and effluents.

Keywords: Estrogen; Estradiol; Ethinylestradiol; Environment; Eco-hormones; Endocrine disruptors

1. Introduction

The presence in our environment of compounds with estrogenic properties has become a major subject of world-wide growing concern, because these compounds may interfere with the reproduction of man, livestock and wild-living animals. As such, recently much research is directed towards the occurrence, effects and risks of these compounds. One of the groups of compounds under investigation are the natural estrogenic hormones, primarily synthesised in the female body and essential for female characteristics and reproduction, and closely related synthetic hormones. The hormones 17β-estradiol and estrone are naturally excreted by women (2–12

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μg/person/day and 3–20 μg/person/day, respectively) and female animals, but also by men (estrone 5 μg/person/day) (Gower, 1975). The majority of the hormones are hydroxylated and conjugated to glucuronides prior to excretion. Desbrow et al. (1998) revealed that in the UK estrogenic activity in some rivers is (at least in part) caused by the presence of 17β-estradiol and estrone, as well as the synthetic birth control contraceptive 17α-ethinylestradiol. Snyder et al. did similar observations in an effluent-dominated river in the USA (results presented at SETAC Conference, San Francisco, November 1997).

A bottle neck in the routine analyses of hormones in surface and waste water is the absence of a sufficiently sensitive analytical procedure. Based on the few measurements available and the relatively small amounts excreted by humans and livestock, it is expected that concentrations of hormones in surface water in The Netherlands will be low. 17β-Estradiol, estrone and 17α-ethinylestradiol have been detected in effluents of British, Israeli and German waste water treatment plants (WWTP) and in surface water in English and German rivers and an Israeli lake in the ng/l range (Aherne and Briggs, 1989; Shore et al., 1993; Stumpf et al., 1996; Desbrow et al., 1998). These studies report the concentrations of biologically active estrogens only and the concentrations of hormone glucuronides in surface or waste water are unknown.

The objective of this study was to develop an analytical procedure that enables routine analysis of four estrogenic hormones and their glucuronides in concentrations below 1 ng/l in surface water and waste water. The second objective of this study was to obtain a first impression of the occurrence of the four hormones and their glucuronides in the Dutch aquatic environment, including selected waste water effluents.

2. Materials and methods

2.1. Materials

17β-Estradiol, 17α-estradiol, estrone, 17α-ethinylestradiol and β-estradiol-17-(β-D)-glucuronide were obtained from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands). All solvents were obtained from Baker and were HPLC pure (methanol and water), ultrares (acetone) or p.a. (ethylacetate and toluene). The silylation agent SIL A (dimethyldichlorosilane solution in toluene) was obtained from Sigma. Filters were from Schleicher and Schuell (0.45 μm) and Wattman (1.2 μm). The polystyrene divinylbenzene extraction disk SDB-XC was obtained from Varian, while the octadecyl (C18) SPE column and amino (NH_2) SPE column were obtained from Baker. Prior to use, all glass work used in the analytical procedure was deactivated with a dimethyldichlorosilane solution in toluene and rinsed with toluene and methanol and the extraction disks were activated subsequently with acetone, methanol and water.

2.2. Sampling of surface water and waste water effluents

In autumn 1997, final treated effluents of five WWTPs (three mainly domestic, two industrial) were sampled (1 l) twice (October and December 1997). All five WWTPs are based on the activated sludge system (either the Aeration tank type or the Carrousel type). The 'domestic' WWTPs have a total capacity of 300 000–560 000 population equivalents, the capacity of the two industrial WWTPs is less than 100 000 population equivalents. The average load is 75–95% of the total capacity. The WWTP-samples were collected over a 7-h period.

In addition, 1-l surface water samples were collected at 11 coastal/estuarine and freshwater locations in The Netherlands that are part of routine water quality monitoring programmes. The sampling locations are shown on the map (Fig. 1). The sampling points include surface water of the two major rivers Meuse and Rhine where they enter The Netherlands at Eysden (location 1) and Lobith (location 2), respectively. Upstream of these sites are the densely populated and heavily industrialised areas in and around Liege in Belgium and The Ruhr area in Germany. The Rhine is also sampled downstream in the major harbour city Rotterdam and in its industrialised vicinity (locations 3 and 4) and downstream

Fig. 1. Location of sampling sites in The Netherlands. 1. Meuse — Eysden; 2. Rhine — Lobith; 3. New Waterway — Beneluxtunnel; 4. New Waterway — Maassluis; 5. North Sea Canal — IJmuiden; 6. Haringvliet; 7. Canal Gent-Terneuzen; 8. Western Scheldt — Terneuzen; 9. Western Scheldt — Hansweert; 10. Eastern Scheldt; 11. Delfzijl sea harbour canal.

of Amsterdam and the industrialised zone of the Port of Amsterdam (location 5). Location 6 receives both water from the Rhine and Meuse, and is situated in a rural area. Location 7, connecting the Belgium town Gent with the Western Scheldt, is a canal with regional importance for the local industry. The outlet of this canal and the river Scheldt are situated in the Delta area (locations 8 and 9), which is in open connection with the North Sea. Also part of the Delta area is the Eastern Scheldt (location 10), which is a relatively clean enclosed marine sea arm. Location 11 is situated in a sea harbour outlet, which is in connection with the Wadden Sea. The land around locations 8–11 is dominated by agriculture and some industry and is sparsely populated.

All samples were collected in green pre-silylated 1-l glass bottles, refrigerated and transported to the lab within 1 day. Most samples were filtered and extracted on extraction disk within another day. The extraction disks, or the eluates of the disks were stored in the freezer until further clean up and analysis. Three locations (locations 1, 2 and 4) were sampled twice with 2.5 months time lapse (August and November 1997). At these three freshwater locations (locations 1, 2 and 4) and at the five waste water treatment plants, additional 1-l samples have been taken in order to analyze the glucuronides of the hormones.

2.3. Analysis of hormones

Based on the methods of Rathner and Sonneborn (1979), Fotsis and Adlercreutz (1987),

Daeseliere et al. (1991), Brooks et al. (1993), Kalbfus (1995) and Stumpf et al. (1996) an analytical procedure for natural hormones was developed that enables routine analyses at low concentrations.

Samples consisting of 1 l surface water or waste water were filtered over combined 0.45-μm and 1.2-μm filters. The compounds were extracted with a acetone and methanol activated SDB-XC disk in a Varian disk extraction apparatus. The compounds were eluted from the disk with 3×5 ml methanol. The methanol was subsequently evaporated at 60°C under nitrogen and the residue (max. 100 μl) was cleaned over a combination of C18 and NH_2 columns (Baker), which were activated with methanol/water and ethylacetate, respectively. The hormones were eluted with 3×1 ml ethylacetate. The ethylacetate was evaporated under nitrogen to dryness at 60°C. The residue was dissolved in HPLC eluent (methanol/water 65/35) and fractionated with HPLC with a 15 cm $\times$ 4.6 mm I.D. S5 PAH column (Phase Separations) with methanol/water 65/35 as the mobile phase flowing at 1 ml/min. The hormones were eluted after $\sim$8–11 min. Fractions containing the hormones were collected, evaporated until dryness at 60°C and silylated with SIL A reagent during 1 h at 60°C. After evaporation, the residue was taken into hexane together with the internal standard PCB103. This mixture was then washed with water in order to remove harmful by-products that contaminate the GC column or detector. The hexane phase was dried over a sodium sulphate column and collected in a GC-vial. The hexane fraction was evaporated to 50 μl of which 3 μl were used for GC-MS/MS.

The formed complex was measured with GC-MS/MS (Varian 3400 GC with a Saturn IV ion-trap mass spectrometer), equipped with a 30-m DB-5MS column (0.25 mm I.D., 0.25 μm film). Since the silylation reaction resulted in aggressive by-products, the GC column was protected with a 2-m retention gap of deactivated fused silica 0.53 I.D. The limit of detection (LOD) was set at three times the noise level of the baseline in the chromatogram, while the limit of quantification (LOQ) was set at three times the LOD. Values between LOD and LOQ are indicated in the tables with an asterisk (*). The LOD varies with the matrix, and therefore there are different LOD for different samples.

The robustness of the analytical procedure was tested with 12 l waste water (effluent). Six times 1 l was used to determine the background levels and the LOD of this sample. The other 6 l were used to determine the recovery and reproducibility in sixfold and were spiked with the four hormones in concentrations of 10 ng/l. All 12 samples were analyzed as described above.

2.4. Analysis of hormone glucuronides

The analytical procedure for hormone glucuronides is similar to that of the hormones described above, except for the deglucuronidation step before analysis. Thus, in this case, total hormones (hormones + hormone glucuronides) were measured. After elution of the extraction disk the methanol was evaporated until dryness and the residue was dissolved in 5 ml 0.2 M sodium-acetate buffer. Fifty μl of the enzyme β-glucuronidase (H2 type, β-glucuronidase activity 100 000 E/ml, sulphatase activity 5000 E/ml) was added and the mixture was left overnight at 37°C. The mixture was extracted with the combination of C18 and NH_2 columns and further cleaned with HPLC as described above. The efficiency of the enzyme reaction was tested with a standard solution of β-estradiol-17-glucuronide, while the recovery of the whole procedure was tested with 1 l surface water spiked with 20 ng/l β-estradiol-17-glucuronide.

3. Results and discussion

3.1. Characteristics of the analytical procedure of the hormones and hormone glucuronides

An overview of the detection characteristics of the four hormones is presented in Table 1. The five point calibration curves were linear in the 2–10 ng/l range. Therefore, all samples were concentrated or diluted in such a way that they fall within this range. The recovery of the procedure is 88–98% ($n = 6$) with an LOD of 0.1–2.4 ng/l (Table 1). Limit of detections in effluents

Table 1
Detection characteristics and characteristics of analytical procedure of four estrogenic hormones

Compound	Retention time on GC-MS/MS (min)	m/z (quantification)	Recovery (%)	Limit of detection (ng/l)[a]	
				Surface water	Effluent
17α-estradiol	23.4	285 + 326	88 ± 12	0.1–0.3	0.1–1.2
Estrone	24.1	242 + 257	98 ± 14	0.2–0.3	0.3–1
17β-estradiol	24.3	285 + 326	88 ± 9	0.3–0.6	0.5–2.4
17α-ethinylestradiol	26.5	193 + 231 + 303	96 ± 8	0.1–0.3	0.3–1.8

[a] Limit of detection varies with the matrix.

are higher than in surface water because the matrix of the former is more complicated.

The recovery of estradiol-17-glucuronide (extraction, enzyme reaction and further analysis as described for the hormones) was 59 ± 3% ($n = 4$). The completeness of the enzyme reaction was tested separately with two replicates of a standard solution of 17β-estradiol-17-glucuronide and proved to be 105 and 116%.

3.2. Occurrence of hormones in treated waste water

In most effluent samples measured in this study, estrone and 17β-estradiol were detected (Table 2). Highest concentrations were measured for estrone (up to 47 ng/l in domestic effluent), while 17β-estradiol was found in concentrations of 1–12 ng/l. The other two hormones 17α-estradiol and

Table 2
Concentration of hormones in five effluents of waste water treatment plants in The Netherlands

Type of WWTP	Sampling location and period	Concentration[a,b] (ng/l)			
		17β-estradiol	17α-estradiol	17α-ethinylestradiol	Estrone
Domestic	WWTP A (Oct. 97)	n.a.[c]	< 1.3 (< 1.3)	< 1.4 (< 1.4)	2.7 (5.4)
	WWTP A (Dec. 97)	1.1*	< 0.1	< 0.2	15
	WWTP B (Oct. 97)	n.a.[c]	< 1.7 (< 1.3)	< 1.8 (< 1.4)	< 0.4 (1*)
	WWTP B (Dec. 97)	0.7*	1.2	< 0.2	6.3
	WWTP C (Oct. 97)	< 0.6 (< 0.6)	< 0.1 (< 0.1)	< 0.3 (0.5*)	2.1 (2.2)
	WWTP C (Dec. 97)	12	5.0	7.5	47
	Median concentration	0.9[d]	< LOD	< LOD	4.5[d]
	Number of locations where compound was detected in Oct. 97	1 of 1	0 of 3	0 of 3	2 of 3
	Number of locations where compound was detected in Dec. 97	3 of 3	2 of 3	1 of 3	3 of 3
Industrial	WWTP D (Oct. 97)	< 0.6 (< 0.5)	< 0.5 (< 0.3)	< 1.8 (< 1.4)	11 (7.4)
	WWTP D (Dec. 97)	1.8	2.1	2.6	0.7
	WWTP E (Oct. 97)	< 0.7 (< 0.6)	< 0.1 (< 0.1)	< 0.3 (< 0.3)	< 0.4 (< 0.3)
	WWTP E (Dec. 97)	< 0.4	< 0.1	< 0.2	< 0.1
	Median concentration	< LOD	< LOD	< LOD	0.4[d]
	Number of locations where compound was detected in Oct. 97	0 of 2	0 of 2	0 of 2	1 of 2
	Number of locations where compound was detected in Dec. 97	1 of 2	1 of 2	1 of 2	1 of 2

[a] Values are not corrected for recovery. Values between limit of detection (LOD) and limit of quantification are indicated in the tables with an asterisk (*).
[b] Values in parentheses refer to the same sample after treatment with β-glucuronidase (for explanation see text).
[c] Not analyzed due to technical problems during HPLC fractionation.
[d] Average of two median values.

17α-ethinylestradiol were only detected at one occasion in three and two effluent samples, respectively. The data also show that concentrations of all hormones were higher in domestic effluents than in industrial effluents.

Levels of hormones in effluent samples treated with the enzyme β-glucuronidase and the matching untreated samples fall in the same range. In two domestic WWTP effluents, estrone levels in enzymatically treated samples were twice as high compared to untreated samples (5.4 ng/l and 2.7 ng/l, respectively and 1 ng/l and <0.4 ng/l, respectively). Only in the first effluent sample mentioned, levels of estrone were above the level of quantification. In the other three WWTP effluents, levels of estrone in treated and untreated samples were identical, as were the other hormones in all five WWTPs. Consequently, it seems that in these cases levels of hormone glucuronides were below detection limit. This was unexpected as the major excretion products of hormones by women are the biologically inactive hormone glucuronides. It is possible that these hormone glucuronides are further degraded or transformed back into hormones. However, this is a hypothetical assumption and more research on the fate of hormone glucuronides is necessary.

Finally it should be noted that there is a clear difference in concentrations in the October and December series. This difference is reflected by a difference in concentrations of the hormones in the influents (Belfroid et al., unpublished results).

In a survey of effluents of German WWTPs, 17α-ethinylestradiol was detected in all 20 WWTPs investigated above the quantification level of 1 ng/l and in 15 effluents >10 ng/l. The median concentration of 17α-ethinylestradiol was 17 ng/l and the maximum 62 ng/l. 17β-Estradiol was detected in eight out of 20 effluents (two effluents >10 ng/l) with a maximum concentration of 21 ng/l (Stumpf et al., 1996). Similar results have been found in Israel and the UK. In Tel Aviv, estrogens were 24–48 ng/l (Shore et al., 1993). In the UK, concentrations in effluents varied from 1 to 50 ng/l estrone, 2 to 50 ng/l 17β-estradiol and up to 7 ng/l 17α-ethinylestradiol (Aherne and Briggs, 1989; Desbrow et al., 1998). According to Kalbfus (1995), however, concentrations of 17α-ethinylestradiol in German effluents were in the range of 0.3–0.5 ng/l (number of WWTPs not reported). The concentrations of hormones measured in effluents in The Netherlands seem to be in the low range or lower compared to the data reported for Germany, Israel or the UK. More research is necessary to clarify these observed differences.

3.3. Occurrence of hormones in surface water

Table 3 summarises the results of the analysis of hormones in surface water samples. Generally, concentrations of hormones in surface water are low (below 1–5 ng/l). Of the four hormones considered in this study, estrone was detected most frequently. As Table 3 shows, highest levels of hormones were found in rivers and canals such as at locations 1, 2 and 4. However, many values are still below the limit of quantification and a consistent pattern is absent, as is clear from the data of locations 2 and 4, where hormones were only detected at one sampling occasion. Concentrations in the samples collected in more open water (locations 6 and 8–11, see Fig. 1) are all below LOD.

Again, as was the case for waste water samples, levels of hormones in samples treated with the enzyme β-glucuronidase are similar compared to the levels in the matching untreated samples (locations 1 and 4). This implies that hormone glucuronides were not present in concentrations above the limit of detection. The only exception are the levels of all four hormones at location 2, which are higher in enzymatically-treated samples compared to untreated samples. However, the levels of hormones in the treated samples are just above the LOD, and still under the LOQ. Therefore, it is difficult to draw any conclusion on this observation.

Few data on the presence of hormones are available in the literature. In the UK, immunoassay detection reveals the presence of 17α-ethinylestradiol in rivers in concentrations below 5 ng/l in September 1982 and 2–15 ng/l in August 1987 (Aherne and Briggs, 1989). In Germany, in The Ruhr district, 17α-ethinylestradiol has been detected in surface water in concentrations between

Table 3
Concentration of hormones in surface water in The Netherlands

Location, sampling date	Concentration[a,b] (ng/l)			
	17β-estradiol	17α-estradiol	17α-ethinylestradiol	Estrone
1. Meuse — Eysden 26/8/97	0.8* (0.9*)	0.3* (0.4)	0.4* (0.3*)	2.7 (2.1)
5/11/97	5.5	1.1	< 0.2	2.5
9/12/97	0.6*	< 0.1	< 0.2	3.4
2. Rhine — Lobith 27/8/97	< 0.6 (0.7*)	< 0.1 (0.3*)	< 0.3 (0.3*)	< 0.3 (0.7*)
11/11/97[c]	1.0*	1.4	1.2	1.7
11/11/97[c]	2.8	3.0	4.3	2.9
3. New Waterway — Beneluxtunnel 2/10/97	< 0.3	< 0.1	< 0.1	0.3*
4. New Waterway — Maassluis 20/8/97	0.6* (0.6*)	0.2* (0.2*)	0.3* (< 0.3)	0.6* (0.8*)
3/11/97	< 0.4	< 0.1	< 0.2	< 0.1
5. North Sea Canal — IJmuiden 1/10/97	< 0.3	< 0.1	< 0.1	0.5*
6. Haringvliet 5/11/97	< 0.4	< 0.1	< 0.2	< 0.1
7. Canal Gent-Terneuzen 24/9/97	0.3*	< 0.1	< 0.1	0.6
8. Western Scheldt — Terneuzen 24/9	< 0.3	< 0.1	< 0.1	< 0.2
9. Western Scheldt — Hansweert 23/9	< 0.3	< 0.1	< 0.1	< 0.2
10. Eastern Scheldt 13/10/97	< 0.3	< 0.1	< 0.1	< 0.2
11. Delfzijl sea canal 14/10/97	< 0.3	< 0.1	< 0.1	0.5*
Median concentration	< LOD	< LOD	< LOD	0.3
Number of locations where compound was observed	4 of 11	3 of 11	3 of 11	7 of 11

[a] Data are not corrected for recovery. Values between LOD and LOQ are indicated in the tables with an asterisk (*).
[b] Values in parentheses refer to the same sample after treatment with β-glucuronidase (for explanation see text).
[c] Two separate samples.

< 1–4 ng/l, while 17β-estradiol and estrone were below the quantification limit of < 1 ng/l (Stumpf et al., 1996). The measurements in The Netherlands show that the situation resembles that in Germany.

4. Conclusion

An analytical procedure has been developed that enables routine analysis of four estrogenic hormones in surface water and waste water with a recovery of 88–98% and a limit of detection of 0.1–2.4 ng/l. Application of the procedure revealed that in The Netherlands estrogenic hormones can be detected at some locations and at certain time points in surface water in concentrations up to 6 ng/l. At other time points and/or locations levels are below the LOD.

In effluents of waste water treatment plants, estrone and 17β-estradiol were detected in concentrations in the ng/l range. Concentrations of 17α-estradiol and the 17α-ethinylestradiol were in most samples below the limit of detection. Most surface water and effluent samples treated with the enzyme β-glucuronidase did not show increased levels of hormones in the matching samples, indicating that hormone-glucuronides were not present in concentrations above the limit of detection. In only one effluent sample, increased levels above the limit of quantification for estrone were observed.

References

Aherne GW, Briggs R. The relevance of the presence of certain synthetic steroids in the aquatic environment. J Pharm Pharmacol 1989;41:735–736.

Brooks CJW, Walash MI, Zakhari NA, Toubar SS, Watson DG. Assay of certain contraceptive formulations by gas chromatography-mass spectrometry-selected ion monitoring. Acta Pharm Hungar 1993;63:19–27.

Daeseliere E, De Guesquière A, Van Peteghem C. Detection of the illegal use of ethinylestradiol in cattle urine by gas

chromatography-mass spectrometry. J Chromatogr 1991; 564:469–475.

Desbrow C, Routledge E, Brighty GC, Sumpter J, Waldock M. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. Environ Sci Technol 1998;32:1549–1558.

Fotsis T, Adlercreutz H. The multicomponent analysis of estrogens in urine by ion exchange chromatography and GC-MS. 1. Quantitation of estrogens after initial hydrolysis of conjugates. J Steroid Biochem 1987;28:203–213.

Gower DB. Catabolism and excretion of steroids. In: Makin HLJ, editor. Biochemistry of steroid hormones. Oxford, UK: Blackwell, 1975:127–148.

Kalbfus W. Belastung bayerischer Gewässer durch synthetische Östrogene. Paper presented at 50. Fachtagung, Bayerisches Landesamt for Wasserwirtschaft, Insitut für Wasserforschung, München, 7/8 Nov. 1995 (in German).

Rathner M, Sonneborn M. Biologische wirksame östrogene in trink- und abwasser. Forum Städte-Hygiene (in German) 1979;30:45–49.

Shore LS, Gurevitz M, Shemesh M. Estrogen as an environmental pollutant. Bull Environ Contam Toxicol 1993;51: 361–366.

Stumpf M, Ternes TA, Haberer K, Baumann W. Nachweis von natürlichen und synthetischen Östrogenen in Kläranlagen und Fliessgewässern. Vom Wasser (in German) 1996;87:251–261.