

Study of Endocrine Disrupting Chemicals in Environment

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Abstract: Endocrine disrupting chemicals (EDC) cause more and more serious environmental pollutions. The EDCs show only ng-µg/l concentration level in the environment, therefore their determinations require multistep sample preparation processes and highly sophisticated instrumentation. This paper discuss the EDC effects, and show examples for determination of such compounds.

Keywords: endocrine disrupting chemicals, toxic effects, sample preparation, GC/MS

1 Introduction

The pollution effects of endocrine disrupting chemicals (EDC) gain more and more attention in worldwide [1-5]. An EDC is exogenous agent, which interferes with the synthesis, secretion, transport, binding action, or elimination of natural hormones in the body, which are responsible for the maintenance of homeostasis, reproduction, development, or behavior. They can cause difficulties in cell, organ, individual, and population levels.

For example, DDT was the reason of broken eggs of predatory birds, resulting in a sharp decrease in their number around the Great Lakes [6]. The symptoms, which had been observed in animal kingdom, are also recognized in mankind. Parents, working in certain pesticide factories, produced girls in significantly higher number, than boys [7].

Such shocking facts have triggered the research in this field. Recognizing the importance of research of EDCs, we have also launched research project in this topic [8, 9].

2 The Endocrine Distrubing Phenomena

2.1 Structure of Endocrine System

The actions of the hormones work along hormonal axis [10]. There are adrenal, thyroid, ovarian hormone axes. Their functions take place in cascade systems. The brain gives signals to hypothalamus through the nervous system. The hypothalamus passes the signal to pituitary with compounds called releasing hormones. The signal reaches the hormone producing organs with hormones. The hormone producing organs synthesize further hormones, which trigger action binding to hormone receptors. The receptor binding action can induce protein synthesis via mRNA in the cell's nucleus. The releasing hormones and hormones are transported by blood stream. The thyroid hormone axe consists of the following: hypothalamus \rightarrow (thyrotropin, releasing hormone) \rightarrow pituitary gland \rightarrow (Thyrotropin hormone) \rightarrow thyroid gland \rightarrow (thyroxine, triiodothyronine hormones) \rightarrow hormone receptors in cells. The major functions of thyroid hormones are the regulation of body temperature, regulation of metabolism, growth, and they have important role in reproductive developments.

The EDCs may disturb the hormone functions: occupying receptor sites, reactions with hormones, modifying the synthesis of hormones, inducing false protein syntheses. The EDC, binding to receptors, induced tumor, caused infertility, decreased immune strength in several occasions [11].

2.2 Establishing EDC Effects of Compounds and Monitoring Them

The recognition of harmful effects of EDCs is rather difficult. Several chemicals can cause EDC effect only for certain species. These materials have accelerated damaged in special life periods (embryo, newborn, pubertal and pregnant). Therefore the establishment of EDC risk needs several generations tests [12, 13]. Moreover several EDS are persistent, and show biomagnifications and bioaccumulations.

According to an EPA survey, 115 chemicals have serious EDC effects, but more than 87000 chemicals have to be tested for it in the near future [13].

The big number of potential EDCs suggest multistep test methods, namely only those compounds go to the more expensive second step, which were positive in the first screening step. Several representative species are involved into the test procedure, because the EDCs can act differently in various species of animals. The various tests target different endocrine cascades. The EPA worked out the following test system [13]:

First step

– *In vitro*

- ER binding / transcriptional activation assay
- AR binding / transcriptional activation assay
- Steroid genesis assay with minced testis

– *In vivo*

- Rodent 3-day uterotrophic assay (subcutaneous)
- Rodent 20-day pubertal female assay with thyroid
- Rodent 5-7 day Hershberger assay
- Frog metamorphosis assay
- Fish gonadal recrudescence assay

Second step

Mammalian tests

- Two generation reproductive toxicity
- An alternative less comprehensive test
 - Alternative mammalian reproductive
 - One-generation test
 - Non- mammalian multigenerational tests
- Avian reproduction
- Fish life cycle
- Mysid life cycle
- Amphibian development and reproduction

The EDCs can be divided groups according to their origins [7]:

- Natural and artificial hormones (e.g. fitoestrogens, 3-omega-fatty acids, contraceptive pills, and thyroid medicines),

- Drugs with hormonal side effects (e.g. naproxen, metoprolol and clofibrate),
- Chemicals of industry and household (e.g. phthalates, alkylphenol ethoxilate detergents, 1,4-dichloro-benzene and PCBs),
- Pesticides (e.g. lindane, DDT, atrazine and tributyl -tin),
- Side product of industrial and household processes (e.g. PAH, dioxin, pentachloro benzene).

Monitoring the EDCs requires highly sophisticated analyses methods, because their concentration levels are very low (pg/l- μ g/l) in the environment [2-4, 8, 9]. Such a low concentration levels need multistep sample cleaning and concentration pretreatments. The used analytical instrumentations are also dedicated (GC/MS, HPLC/MS, CE/MS). Some monitoring tests, based on immunoassay (e.g. Western blot) are also known for monitoring purposes [14]. The exact determination of EDCs requires sometimes even chiral selective analytical methods [15].

3 Determination of some EDCs

The authors present their analytical results for some EDCs from drinking water, natural water (Danube), waste water (Southern-Pest waste water plant).

3.1 Determination of Alkyl Phenols

The alkylphenols ethoxylate, non-ionic detergents have serious estrogenic EDC effects [3, 7, 9]. They are in the list of European Water Framework Directives [16], but these compounds have not established, standard analytical method yet. We developed and validated analytical method for nonyl- and octyl- phenols, the persistent metabolites of etoxylated mother compounds.

3.1.1 Experimental Conditions for Determination Alkyl Phenols

The developed method has been summarized in Figure 1.

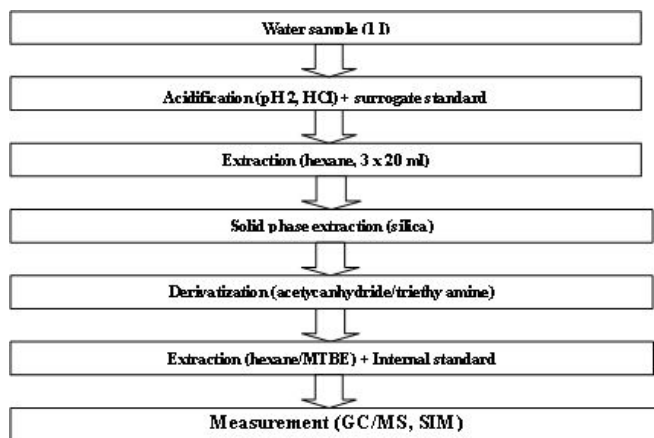


Figure 1

Flow chart of analytical procedure for determination of alkylphenols from water matrices

3.1.2 Results of Analyses of Alkylphenols

The acidification of water sample was necessary to achieve 40-50% recovery, bringing phenols in their undissociated forms. The hexane is advantageous because it has low toxicity, lighter than water and relatively cheap. 2,6-di-*tert*-butylphenol was chosen for surrogate standard, because its chemical structure is similar to the compounds of interests, and it is a very rarely polluting material.

The silica gel solid phase extraction (SPE) cleaning step made the samples free from apolar triglyceride and hydrocarbons with hexane washing step. The dichloromethane elution gave good recovery, and lived the polar humic and protein dirt on adsorber.

Derivatization was necessary to reach 5 ng/l quantification limit. The underivatized phenols showed 20 times higher quantification limit than derivatized ones. The standard acetylating procedure [17] proved the best, using acetic anhydride with triethylamine catalyses. The yield of acetylation was above 95% after 1 hour reaction (60°C).

The unreacted components and side products destroy the filament of mass spectrometer, therefore the derivatized compounds were extracted from water diluted reaction mixture. Hexane: methyl-*tert*-butyl ether (4:1) extraction medium was the best with > 90% yields. Terphenyl internal standard was added to solution before GC/MS measurements.

The GC/MS instrument was used in selected ion monitoring mode to improve the sensitivity. The retention times and mass fragments of sample were identical with the alkyl phenol standards. A representative analysis of raw waste water is in Figure 2.

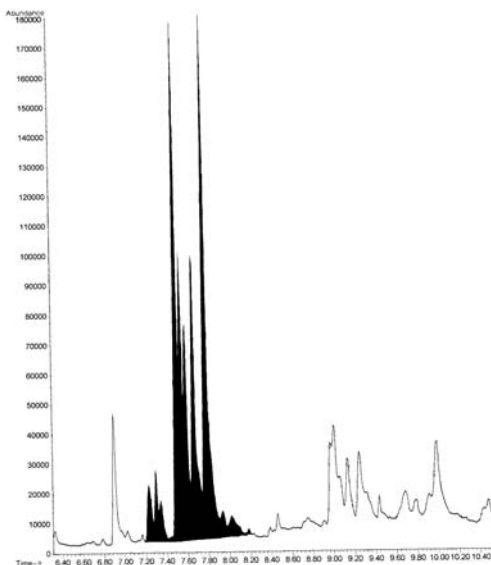


Figure 2

GC/MS analysis of alkylphenols content of raw waste water (Southern-Pest waste water plant)
 Conditions: GC/MS Instrument, HP 5890/HP5790; column, 20 m x 0.18 FSOT, DB-5MS; injection, splitless (1 min); temperature program, 60°C → 300°C (20°C/min); carrier, He (45 kPa); ion source, 70 eV, SIM, 135 m/z.

The calibration curve was linear (r^2 0.995) in the 5- 5000 ng/l range.

Every tested water sample contained alkyl phenols (Table 1).

Table 1
 Alkylphenol content of various water samples

Sample	Alkylphenol content (ng/l)
Drinking water	290
Danube water	320
Treated waste water	1000
Raw waste water	16500

3.2 Determination of Naproxen

Naproxen 2-(6-methoxynaphthalen-2-yl)propanoic acid is a widely used non-steroidal anti-inflammatory drug (NSAID). 6.8 tons of this substance were sold in Hungary (1998) in various formulation. The naproxen has EDC effect disturbing the synthesis of prostaglandins [2]. None of the pharmaceutical compounds had been analyzed in environment in Hungary, therefore we launched

a project to start such research. We have chosen naproxen as starting compound, because its consumption is rather high, its metabolism moderate, and having EDC effect [8].

3.2.1 Experimental Conditions for Determination of Naproxen

The developed analytical process is summarized in Figure 3.

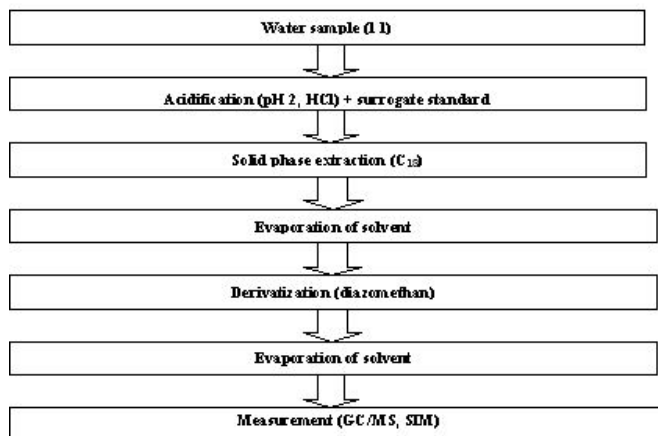


Figure 3

Flow chart of analysis of naproxen from various environmental matrices

3.2.2 Results of Analyses of Naproxen

The β -naphtholic acid proved a good surrogate standard, because its chemical features are rather similar to naproxen, and it is almost impossible to find it in environmental samples.

It was necessary to decrease the pH value of water to bring naproxen into its unhydrolyzed form. Namely, the unhydrolyzed form of acids (naproxen, β -naphtholic acid) can be trapped on a polar adsorbent (C₁₈).

The washing steps with water elute the polar (e.g. proteins, amines) matrix compounds from the disk. The elution had 32 \pm 5% yield using methanol and acetone as elution solvents. Unfortunately, the selectivity of the C₁₈ disk is moderate toward acidic compounds. However, the majority of apolar matrix compounds remained (e.g. hydrocarbons, triglycerides) on the disk. The disk form of adsorbent is better than column form, because the columns can easily be clogged by solid particles of waste water. Moreover, the waste water sample had to be prefiltered through a 0.45 μ m glass membrane.

The moderate selectivity of C₁₈ absorber required 20 ml elution solvent, which were evaporated before the next step.

The underivatized acidic compounds are polar and have low volatility and strong adsorption characters. These feature do not suit well to gas chromatographic process, therefore the acids are derivatized before their GC analyses. Standard methylation with diazomethane [17] was chosen to produce methyl ester derivative of naproxen. The methyl ester derivative of naproxen proved idle for gas chromatography, and produced well recognizable fragments for mass spectrometric quantification.

Deuterated compound, D₁₂flouranthene was used as internal standard according to practice of our laboratory.

The gas chromatographic parameters were optimized, using HP 5890 II instrument, with 30 m x 0.25 mm FSOT columns, coated by DB-5ms stationary phase. To improve the sensitivity, splitless injections were (1 min) applied. Temperature program (50°C /2 min/→10°C/min →300°C), and the carrier gas (15 psi) was used to get sharp, well resolved peaks.

High resolution mass spectrometer (VG AutoSpec-S) was on-line connected to GC in electron impact mode (70 eV). The following m/z values were measured: 185.096 (naproxen), 186.068 (β-naphtholic acid), 244.11 (naproxen) and 214.142 (D₁₂flouranthene). The given m/z values are quantified at the time windows, determined by the retention times of observed compounds.

The calibration curve was linear ($r^2:0.988$) in 1-400 ng/l range. The naproxen content of various water samples are summarized in Table 2.

Table 2
The found naproxen concentration in various water media

Sample	Naproxen content (ng/l)
Drinking water	Non detectable
Danube water	2.4
Treated waste water*	87.5
Raw waste water*	250

*Southern-Pest waste water plant

We have found naproxen in Danube, waste waters, but not in drinking water.

Conclusions

Appropriate analytical methods has been work out for alkylphenol compounds, which are compounds to be monitored on the water framework directives. Every tested matrix contains alkylphenol well above 100 ng/l level. The efficiency of waste water cleaning is poor from point of view of alkylphenols.

Naproxen has found in Danube and waste water, but non in drinking water. The cleaning efficiency of observed waste water plants are moderate toward to naproxen.

Creating these analyses method prepared our laboratory for the analyses of endocrine disrupting chemicals and pharmaceuticals even at ng/l concentration level.

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