Determination of steroids, as their trimethylsilyl-(oxime)-ether/ester derivatives, in the dissolved and in the suspended phases of wastewater and Danube River samples, by gas chromatography-tandem mass spectrometry

PhD thesis

Nóra Andrási, Pharm.D.

Pharmaceutical Sciences Doctoral School
Semmelweis University

Supervisor: Ibolya Molnár-Perl, Ph.D., D.Sc.

Official reviewers: Krisztina Ludányi, Ph.D.
Mária Mörtl, Ph.D.

Head of the Final Examination Committee: Imre Klebovich, Ph.D., D.Sc.

Members of the Final Examination Committee:
Krisztina Novák-Takács, Ph.D., D.Sc.
Krisztina Kurin-Csörgei, Ph.D.

Budapest, 2012

1. Introduction

The presence of pharmaceuticals in environmental waters has been known for the past 35 years. The fate of these substances in the aquatic environment has obtained increasing attention in the last two decades.

Pharmaceutical substances leave the human body, and reach the wastewaters, in original or metabolized form. Treatment processes applied at the most wastewater treatment plants were not designed in order to eliminate organic micropollutants of the low ng/L ranges. According to one of the first studies published on the subject in 1998, wastewater treatment plants in Germany reduced the pharmaceutical content of the influent wastewater by 60% in average. However, removal efficiency could be as low, as 7% (carbamazepine) or as high as 99% (salicylic acid). The same German research group managed to determine steroid hormones, estrogens in river water. Conjugated metabolites can be digested by bacteria during the treatment processes, thus elevating the concentration of the unconjugated form. Several studies were undertaken, with the support of the European Union, to assess the pollutant removal efficiency of the wastewater treatment processes. Based on these studies, 100-3500 ng/L of pharmaceutical compounds and other chemicals are present in effluent wastewaters. These concentrations decrease to 2-400 ng/L due to dilution in river waters. These compounds, and steroid hormones in particular, pose a threat to aquatic ecosystems. Selective and sensitive analytical methods are needed to determine steroids in low ng/L concentrations of water samples- Thus, permitting the tracking of these substances in the aquatic environment, and the development of more efficient wastewater cleaning methods.

The goal of my work was, to insert the investigated steroid compounds into a previously developed, multiresidue analysis procedure, suitable for the analysis of 63 micropollutants from one solution, by a single injection, as their trimethylsilyl (TMS)-oxime ether/ester derivatives, by gas chromatography–mass spectrometry.

2. Objectives

According to the preliminary literature overview, the goals of my PhD work were:

a. to extend the previously developed, multiresidue analysis procedure, with steroid compounds of different types and origin,

b. to perform a detailed derivatization and fragmentation study,

- comparing the response values of the trimethylsilyl-oxime-ether derivatives to the only trimethylsilyl-ether or underivatized ones,
- to collate the derivatization protocols applying the most popular silylating reagents (BSTFA, MSTFA, MTBSTFA) and HMDS+TFA, by the analysis of model solutions, prepared under the same conditions, followed by the optimization of the procedure regarding reaction time and temperature,
- to give a detailed overview on the fragmentation pattern analysis of 20 selected steroids as their TMS (oxime) ether derivatives,

c. to document the reproducibilities of the TMS (oxime) ether derivatives of the selected steroids, along with the corresponding limit of quantitation values from model solutions,

d. to confirm the practical utility of the suggested protocol, by an overview of the steroid contents of the influent and effluent wastewater samples obtained from two Hungarian wastewater treatment plants,

e. to improve the selectivity of the method, by developing MIM and MRM acquisition protocols for the analysis of steroids and cholic acids eluting in the same tierce of the multiresidue analysis system,

f. to perform a critical comparison of the three acquisition techniques (the FS, the MIM and the MRM ones), based on the analysis of the same matrix free solutions and wastewater samples under the same instrumental conditions,

g. to develop a simple, time, cost, labor efficient and quantitative method for the analysis of steroids existing in the suspended phases of target waters, thus defining their distribution between the dissolved and suspended phases of the analyzed wastewater and Danube river samples.

3. Experimental

3.1. Samples

Wastewater samples were taken at the South-Pest Wastewater Treatment Plant of the Budapest Sewage Works Pte. Ltd., and the experimental sewage treatment plant of Organica Water, Inc. in Telki, Hungary. Danube river samples were taken close to the North Building of the Eötvös Loránd University, Faculty of Science.

3.2. Instrumentation

3.2.1. Materials

Glass micro-fiber filters (GF/A 125 mm, ∅, Cat No 1820-125) were from Whatman (Maidstone, UK). Cartridges (Oasis, HLB 6cc), for solid phase extraction (SPE), were from Waters (Milford, MA, USA). SPE extractions were performed on the Visiprep DL Vacuum Manifold for 12 samples (Cat No. 57044) from Supelco (Bellefonte, PA, USA). Ultrasonic extractions were performed on the Bandelin Sonorex (RK 52 H) apparatus (Bandelin electronic, Berlin, Germany.

3.2.2. Gas-chromatography

The apparatus consisted of a Varian 240 GC-MS/MS system (Varian, Walnut Creek, CA, USA), equipped with a Varian CP-8400 Autosampler, and with the Septum-equipped Programmable Injector (SPI). Injections were made using "on column" injection, the column used was a product of SGE (Victoria, Australia); SGE forte BPX5 capillary: 30m x 0.25mm; df = 0.25μm. Helium of 6.0 purity, as carrier gas, had a flow rate of 1 mL/min.

3.2.3. Mass spectrometric conditions

The general MS/MS parameters were: Fil/Mul delay: 20.00 min; mass defect: 0 mmu/100 μ; filament current: 40 μ A; Target TIC: 5000 counts; Prescan Ion Time: 1500 μs; Scan mode: Fast; Scan Time: 0.17 s/scan; Multiplier offset: Autotune + 300 V; electron energy: 70 eV. Ion preparation method (IPM) parameters in each segment were: isolation window: 3.0 m/z; ionization storage level: 35 m/z; high mass ejection: 35 V; excitation time: 20 ms; Modulate RF: yes; Frequency Number: 1, CID frequency offset: 0.0 kHz.

The temperature of the transfer line, ion trap and manifold were, in order of listing 300 °C, 210 °C and 80 °C, respectively.

3.3. Methods

3.3.1. Reagents

The 2.5% (w/v) hydroxylamine-hydrochloride reagent, used for oximation, was prepared by the solution of 0.625g hydroxylamine-hydrochloride in 25mL pyridine. The solution was stored, for a maximum of four weeks, in a refrigerator.

Silylating agents HMDS+TFA, MSTFA, BSTFA, and MTBSTFA, were of analytical grade and used without further purification.

3.3.2. Standard solutions

Model compounds (10 mg/10 mL), weighed with analytical precision, were dissolved in ethanol. Model solutions (10–500 μL) and the extracts were rotary evaporated to dryness at 30–40°C.

3.3.3. Derivatization

3.3.3.1. Trimethylsilyl derivatives

Residues of standard solutions were treated with 125 μL pyridine + 225 μL HMDS + 25 μL TFA and heated in oven, at 70°C for 90 minutes.

3.3.3.2.Trimethylsilyl-oxime-derivatives

Residues of standard solutions were treated with 125 μL hydroxylamine-hydrochloride containing pyridine and heated in oven, at 50°C , at 70°C, or 90°C for 30, 60, 90 minutes.

Solutions cooled to room temperature were treated with 225 μL HMDS + 25 μL TFA, or 250 μL BSTFA, or 250 μL MSTFA or 250 μL MTBSTFA. Mixtures prepared with HMDS+TFA were heated at 50°C, at 70°C, or 90°C for 60, 90, 120 minutes, while solutions of BSTFA, MSTFA and MTBSTFA at 70°C for 90 minutes.

1 μL of the diluted solutions was injected into the GC–MS system.

3.3.4. Sample preparation

To separate steroids present in the suspended and in the dissolved phases of water samples, appropriate aliquots (0.5 L or 1.0 L for wastewater, 3 L, 5 L or 10 L of Danube River water) of the homogenized samples were filtered on GF/A 1.6 μm pore sized glass microfiber filter papers previously weighed with analytical precision.

3.3.4.1. SPE extraction of dissolved phase

Oasis HLB 6 mL (200 mg) SPE cartridges, prior to extraction were conditioned with 5 mL hexane, 5 mL ethyl acetate, 10 mL methanol and 10 mL distilled water. Filtered aliquots (0.5 or 1.0 L of wastewater, 3L, 5L, 10L of Danube water) were adjusted to pH 4 with hydrochloric acid. Extractions, three parallels each, were followed with a flow rate of 4–5 mL/min. Cartridges have been dried by vacuum and elution was performed, in order of listing with 5 mL hexane, 5 mL ethyl acetate and 10 mL methanol.

3.3.4.2. Ultrasonic extraction of suspended solid phase

Glass microfiber filter papers were dried on ambient temperature, cut to 5 x 5 mm pieces and put in glass beakers (150 mL). Extractions were made with a solvent mixture of hexane/ethyl acetate/methanol 1/1/2 (v/v%) (applying the same solvent ratios as used for the SPE process). At first, 40 mL of solvent mixture was added to the glass beakers and sonicated for 20 minutes. This step was repeated two times with 20 mL solvent mixture. Solvent portions were filtered on glass micro-fiber paper, unified and treated the same way as the eluents of the SPE process.

The unified eluents of both the SPE and the ultrasonic extraction process were reduced in volume, evaporated to dryness by means of a rotary evaporator {Büchi Rotavapor R-200 and Büchi Vacuum pump V-700, both from Büchi (Flawil, Switzerland)} at 30–40 ^{0}C (further on: extract).

3.3.4.3. Sample derivatization

The extract residues were treated with 125 μL hydroxylamine hydrochloride containing pyridine, heated in oven at 70 ^{0}C for 30 min. Thereafter silylation was continued with 225 μL HMDS + 25 μL TFA and heated at 70 ^{0}C for 90 min. Samples were taken for the analysis after dilutions with HMDS, 1 μL of the diluted solutions was injected into the GC-MS system. Measurements were carried out using the FS, the MIM, and the MRM acquisition techniques.

4. Results

4.1. Conclusions of steroids' derivatization studies

- The previously developed multiresidue analysis procedure has been extended with steroid compounds of different types and origin. These were natural androgens (androsterone, transdehidroandrosterone, transandrosterone, dihidrotestosterone, testosterone, 4-androstene-3,17-dione), natural estrogens (β-estradiol, estriol), synthetic estrogens (mestranol, ethinylestradiol), synthetic progestogens (norethisterone, gestodene, levonorgestrel, etonogestrel, medroxyprogesterone-acetate), the natural progestogene (progesterone), fecal sterols (coprostanol, cholesterol), and phytosterols (stigmasterol, β-sitosterol), a total of twenty compounds.
- In cases of the keto group(s) containing steroids, without exception, the two step derivatization protocol (1: oximation, 2: trimethylsilylation) proved to be of primary importance: ketosteroids do form TMS (oxime) ethers. The response values of the TMS (oxime) ether derivatives compared to the TMS ether ones, show considerable advantages, in all cases tested, indicating the values of ≥ 1, varying between 1.40 (gestodene) and 4.56 (progesterone).
- Fragmentation characteristics of steroid derivatives showed correlation with the original steroid structure. The TMS-(oxime)-ether derivatives of hydroxy- and 3-ketosteroids proved to be stable, providing molecular ions and fragment ions formed by the loss of one methyl group as abundant ions; while the 17 ketosteroids' mass spectra are characterized by fragment ions originating from the cleavage of the steroid skeleton.
- The responses of the TMS (oxime) derivatives of steroids, gained applying the most popular silylating reagents (BSTFA, MSTFA, MTBSTFA) and HMDS+TFA, proved to be more or less comparable. Based on experiments carried out by changing reaction time and temperature vice versa, both in the cases of oximation and silylation, it can be stated that all conditions applied (50°C, 70°C, 90°C, 30, 60, 90 minutes) resulted in acceptable results.
- Accordingly, and taking account of previous experiences, HMDS+TFA as silylating reagent, and 70°C, 30 min for oximation, 70°C, 90 min for silylation were chosen as optimal derivatization conditions.
- LOQ values, obtained by analyzing eight different amounts between 1.88-750 ng/L of steroid-trimethylsilyl-(oxime)-ethers, from matrix free solutions, were between 1.88-37.6 ng/L.

4.2. The steroid content of two Hungarian wastewater treatment plants' influent and effluent samples

- Recoveries, characterized with the relative standard deviation percentages (RSD%), obtained from fortified effluent wastewater samples (added amounts of steroids ranged between 1–2 μg/L), varied between 79% (mestranol) and 106% (etonogestrel), with an average recovery of 95%. The low solubilities of coprostanol, cholesterol, stigmasterol and β -sitosterol resulted in their low average recovery (34%).
- Under a 10 month period (from December 2009 to September 2010) influent and effluent wastewater samples from two WWTPs (Dél-Pest, Telki) have been analyzed.
- Of twenty steroids investigated, nine were detected in the wastewaters. Concentrations of androgens ranged between 0.74-4.28 μg/L (androsterone), 0.138-4.00 μg/L (transandrosterone) and 0.058-4.50 μg/L (androsterone-3,11-diol-17-one) in influent samples, while in effluent samples they, were below LOQ. β-estradiol and estriol were detected in one influent sample, only, in 0.100 μg/L and 0.054 μg/L concentrations, respectively. High coprostanol (20.0-302 μg/L) and cholesterol (6.7-47.3 μg/L) levels were found in all samples. Stigmasterol and β-sitosterol, because of their low water solubility and moderate response characteristics were found in the overwhelming part of samples below their LOQ values.
- Androsterone-3,11-diol-17-one was identified as its di-TMS-oxime-ether derivative, based on the previous fragmentation studies.

4.3. The role of the acquisition methods in the analysis of natural and synthetic steroids and cholic acids by gas chromatography–mass spectrometry

- Optimized MIM and MRM acquisition methods were proposed, for the determination of steroids and the cholic acids sharing the same time window (lithocholic acid, cholic acid, chenodeoxycholic acid, ursodeoxycholic acid, 3-hydroxy-7-ketocholanic acid).
- The FS, the MIM and the MRM type acquisition methods, all three at once, have been compared, under the same (derivatization and instrumental) conditions, and characterized with the same, comparable analytical performance parameters. Data obtained revealed that, data of six point calibration, both the regression coefficients, and the RSD% values of six injections, proved to be independent on the acquisition methods. LOQ and ILQ values showed considerable differences: the lowest ones proved to be associated with the MRM technique.
- Beside the low LOQs, the most favorable S/N values also confirmed the MRM protocol as the most selective one.

- The suitability of different acquisition techniques, in the cases of five influent and the corresponding effluent wastewater samples, were compared based on the same derivatization and instrumental conditions. On the basis of all results compared, it can be stated, that significant selectivity, sensitivity and reliability enhancement was achieved using the MRM instead of the FS and/or MIM mode, in particular and most importantly in the analysis of β -estradiol, ethinylestradiol and estriol. The advantage of the MRM technique over the MIM one is demonstrated with the considerable overestimation of the β -estradiol (156-1325%) and the ethinylestradiol (582-831%) contents of wastewater samples.

4.4. Determination of steroids in the dissolved and in the suspended phases of wastewater and Danube River samples

- A simple, time, cost, labor efficient and quantitative ultrasonic extraction method was presented for the extraction of steroid pollutants existing in the suspended phases of environmental water samples
- The efficiency of the ultrasonic extraction process was verified by quantifying the suspended steroid content of an influent wastewater sample, in the consecutive ultrasound assisted solvent portions, and by determining steroids' concentrations in the suspended phases, obtained from different sample volumes (0.5L,1.0L of influent waste water, 3L - 10L of Danube River samples).
- Steroids quantified in the suspended phases of wastewaters, proved to be higher in comparison to the amounts determined in their corresponding dissolved ones. The natural estrogen β-estradiol showed up from 0.0049 to 0.0322 μg/L, while fecal sterols (coprostanol, cholesterol) and phytosterols, (stigmasterol and β-sitosterol) manifested considerable higher amounts: coprostanol varied from 9.3 μg/L to 488 μg/L, cholesterol from 0.79 to 427 μg/L, stigmasterol from 0.173 μg/L to 48.5 μg/L and β-sitosterol from 0.415 μg/L to 130 μg/L.
- In the dissolved phases of Danube river samples, coprostanol, cholesterol and β-sitosterol were detected in all cases, while β-estradiol and cholic acid in three, ethinylestradiol in two, stigmasterol in only one sample. Their concentration ranged between 0.330-0.449 μg/L (β-estradiol), 1.14-1.16 μg/L (ethinylestradiol), 18.6-42.1 μg/L (coprostanol), 88-170 μg/L (cholesterol), 23.3 μg/L (stigmasterol), 8.1-128 μg/L (cholic acid) and 128-379 μg/L (β-sitosterol).
- Suspended phases of all Danube river samples contained coprostanol, cholesterol and β-sitosterol while ethinylestradiol and stigmasterol in three cases out of four were

found. The concentration of these steroids ranged between 0.352-0.461 μg/L (ethinylestradiol), 218-263 μg/L (coprostanol), 14.5-525 μg/L (cholesterol), 18.3-179 μg/L (stigmasterol) and 22.3-1780 μg/L (β-sitosterol).

- The overwhelming part of steroids, expressed in their total amounts determined, was found in their suspended phases (71% from WWTPs, 64% from Danube River samples).

5. Summary

The previously developed multiresidue analysis procedure has been extended with 18 natural and synthetic steroids, permitting the identification and quantification, in total of 81 pollutants from one solution, by a single injection, as their (TMS)-oxime ether/ester derivatives, by GC–MS(/MS).

At the basic research level, detailed derivatization optimization studies were performed. Results confirmed, that:

- in order of reliable identification of the hydroxy- and ketosteroids, the two-step derivatization protocol (1. oximation, 2. trimethylsilylation) is obligatory.
- Regarding the derivatization conditions, response values, gained by varying the silylating agents, reaction time and –temperature, proved to be commensurable. The suitability of HMDS+TFA reagent has been verified.

Fragmentation characteristics of steroid derivatives showed correlation with the original steroid structure. The TMS-(oxime)-ether derivatives of hydroxy- and 3-ketosteroids proved to be stable, providing molecular ions and fragment ions formed by the loss of one methyl group as abundant ions; while the 17 ketosteroids' mass spectra are characterized by fragment ions originating from the cleavage of the steroid skeleton.

MIM and MRM tandem mass spectrometric acquisition protocols have been optimized, for the determination of 20 steroids, along with the cholic acids sharing the same time window, for the first time. As novelty to the field, the FS, MIM and MRM techniques have been compared, by applying all three acquisition methods, in parallel, for the analysis of matrix free solutions and wastewater samples. Results confirmed the high selectivity of the MRM acquisition method, even in comparison to the MIM one:

- determination of the *β*-estradiol, ethinylestradiol and estriol contents of wastewaters proved to be reliable, applying the MRM acquisition protocol, only.
- The MRM technique is essential in avoiding the overestimation of wastewater samples' ethinylestradiol content.

The steroid content of Danube and wastewater samples' suspended phases, applying the ultrasound assisted extraction method, has been analyzed with their dissolved counterparts, simultaneously. Results confirmed that the overwhelming part of the water samples' steroid content is present in the suspended phase, isolated by filtration as part of the sample preparation, and usually discarded. Thus, the total amounts of steroids that the ecosystem is liable to, were defined.

Publications:

1. Andrási N, Helenkár A, Záray Gy, Vasanits A, Molnár-Perl I. (2011) The role of the acquisition methods in the analysis of natural and synthetic steroids and cholic acids by gas chromatography–mass spectrometry. J Chromatogr A, 1218: 8264-8272.
2. Andrási N, Helenkár A, Záray Gy, Vasanits A, Molnár-Perl I. (2011) Derivatization and fragmentation pattern analysis of natural and synthetic steroids, as their trimethylsilyl (oxime) ether derivatives by gas chromatography mass spectrometry: Analysis of dissolved steroids in wastewater samples J Chromatogr A, 1218: 1878–1890.
3. Perlné Molnár Ibolya, Zsigrainé Vasanits Anikó, Sebők Ágnes, Helenkár András, Andrási Nóra, Faludi Tamás, Molnár Borbála, Záray Gyula (2012): Környezeti vizek szerves szennyezőinek azonosítása és mérése, trimetilszilil (oxim) éter/észter származékokként, a gázkromatográfia tömegspektrometria felhasználásával Hungarian Journal of Chemistry, 2-4: 55-64.
4. Andrási N., Molnár B., Vasanits-Zsigrai A., Záray Gy., Molnár-Perl I. (2013): Determination of steroids in the dissolved and in the suspended phases of wastewater and Danube River samples by gas chromatography, tandem mass spectrometry Talanta, 115: 367–373.