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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

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ABSTRACT

This paper reports the extension of our multiresidue analysis (MA) procedure 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 trimethylsilyl (TMS)-oxime ether/ester derivatives, by gas chromatography–mass spectrometry (GC–MS), within 31 min. As a novelty to the field, basic researches, such as fragmentation pattern analysis and derivatization optimization studies were performed for androsterone, transdehydroandrosterone, transandrosterone, mestranol, dihydrotestosterone, ethinylestradiol, testosterone, norethisterone, estriol, 4-androstene-3,17-dione, gestodene, levonorgestrel, etonogestrel, coprostanol, progesterone, cholesterol, medroxy-progesterone-acetate, stigmasterol and β-sitosterol. Results confirmed that (i) the TMS oxime-ether derivatives of the keto steroids provide from 1.40 times (gestodene) up to 4.25 times (norethisterone) higher responses compared to their TMS-ether ones, and (ii) the distribution of syn/anti oximes is characteristic to the ketosteroid species examined. Based on our optimized mass fragmentation, solid phase extraction (SPE) and derivatization studies separations have been performed in the total ion current (TIC) mode, identification and quantification of compounds have been carried out on the basis of their selective fragment ions. Responses, obtained with derivatized standards proved to be linear (hydroxysteroids), or have been calculated from calibration curves (ketosteroids) in the range of 1.88–750 ng/L levels. Limit of quantitation (LOQ) values varied between 1.88 ng/L and 37.5 ng/L concentrations. The most important practical messages of this work are the high androsterone (0.744–4.28 μg/L), transandrosterone (0.138–4.00 μg/L), coprostanol (2.11–302 μg/L), cholesterol (0.308–41 μg/L), stigmasterol (1.21–8.40 μg/L) and β-sitosterol (1.12–11.0 μg/L) contents of influent wastewaters. β-Estradiol (100 ng/L) and estriol (54 ng/L) were found in one influent sample, only. Reproducibilities, characterized with the relative standard deviation percentages (RSD%) of measurements, varied between 1.73 RSD% (β-estradiol) and 5.4 RSD% (stigmasterol), with an average of 4.82 RSD%.

1. Introduction

Gas chromatography mass spectrometry (GC–MS) of steroids is still a challenge for analytical chemists. Publications selected for the literature overview (except one [1]), appeared in the last decade [2–77].

The relevancy of the topic can be characterized by the fact that steroid profiling proved to be of primary importance in the diagnosis of clinical disorders [4,11,13–22,26,29,50,53,54,64], in the recognition of drug abuses in sports doping control [8,12], in food analysis [2,3,45] and most importantly in the pollutant analysis of environmental water samples [1,5–7,10,23–25,27,28,30,32–44,46–48,52,53,61–63,65–68]: in this last context case studies confirm the unambiguous harm of steroids impairing wildlife [57–60].

As to the review papers [61–64] – comparing the advantages and disadvantages of the relevant GC–MS/(MS) and LC–MS/(MS) steroid analysis protocols – it seems to be clear that GC–MS/(MS) is at least comparable [61–63], however out and away the method of choice [64]. In agreement with the conviction of the present papers' authors [65–68], GC–MS has been characterized very recently as "... a pre-eminent discovery tool in clinical steroid investigations even in the era of fast liquid chromatography tandem mass spectrometry..." [64].

The literature overview of steroids' derivatization techniques reveals that in the overwhelming part of proposals the use of various silylating reagents has been preferred, like *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) [1–21], bis-

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(trimethylsilyl)trifluoro-acetamide (BSTFA) [22–45], *N*-methyl-*N*-*tert*-butyldimethylsilyl-trifluoroacetamide (MTBSTFA) [46,47], and trimethylsilylimidazole(s) (TMSI) [48–50]. Acylations were performed with pentafluorobenzoyl chloride [51–53] or with heptafluorobutyric anhydride [54,55]. Subsequently to enzymatic oxidation steroids were determined also as hydrazones [56].

In order to define methodological pitfalls selected analytical techniques have been compared, focusing in particular to the optimum silylation conditions of steroids [69–77]. Evaluating the details of these comparisons it turned out that the main uncertainties are associated with the stability of derivatives, depending

(a) on the silylating agents, like MSTFA, BSTFA, and MTBSTFA [69,70,73–76],
(b) on the time and temperature (60 °C, 30 min [69,72,73], 65 °C, 30 min [74], 50 °C, 30 min [71], 85 °C, 100 min [70], microwave: 900 W, 1 min [75], 80 °C, 60 min [76], 60–70 °C, 30 min [77]),
(c) on the optimum solvent of derivatizations, and
(d) on the acquisition protocols applied (GC–MS, GC–MS/(MS) [65]).

Authors of this paper are convinced that

(1) unsatisfactory analytical attention was paid to the distinction, consequently, to the simultaneous identification and quantification of the keto, the keto and hydroxyl and the only hydroxyl group(s) containing steroids, from a single chromatographic run, in shortage of exhaustive mass fragmentation studies,
(2) in several proposals the keto groups' derivatizations are simply neglected [5–7,10,13,16,17],
(3) in others, by means of reductive silylation {MSTFA/NH_4J/dithiothreitol (DTE) ≈ 500–1000/4/2 (v/v/v)}, keto groups were transformed to the corresponding hydroxyl groups containing species: consequently, for sake of distinction, two derivatizations (a reductive and a non reductive one) would be needed [1–4,6,8,11,12,14,15,18–21,60],
(4) the advantage of the analysis of the methyloxime trimethylsilyl derivatives of steroids was, unfortunately, used in few cases, and without basic studies, only [32,48–50,64]. This protocol was applied in the analysis of faecal sterols from catchment waters [32], selected steroids from wastewaters [48], to identify dehydroepiandrosterone and its 7-oxygenated metabolites in human serum [49], to quantify urinary steroids, selectively [50] and to define steroid disorder metabolomes [64].

The goal of this paper was

(1) to give a detailed overview on the fragmentation pattern analysis of 20 selected steroids as their TMS (oxime) ether derivatives applying the optimum two step derivatization protocol (1: oximation; 2: silylation), on basic research level; documenting also the response of the only trimethylsilylated ethers,
(2) to compare derivatization protocols of steroids with the commonly used reagents (MSTFA, BSTFA, MTBSTFA), including the preferred, of our longstanding hexamethyldisilazane + trifluoroacetic acid (HMDS + TFA) one,
(3) 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, and
(4) 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 Waste Water Treatment Plants (WWTPs).

2. Experimental

2.1. Instrumentation

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). The column used was a product of SGE (Victoria, Australia); SGE forte capillary: 30 m × 0.25 mm; df = 0.25 μm. The temperature of the transfer line, ion trap and manifold were, in order of listing 300 °C, 210 °C and 80 °C, respectively.

MS conditions: Electron energy was 70 eV; multiplier offset was 250 eV. The actual parameters of the ITD were defined by the automatic set up mode.
Actual automatic set-up conditions: Mass range: 40–650 amu; the scan rate: 1 scan/second.
Acquisition time: 31 min; solvent delay: 420 s (omitting the acquisition of reagent peaks); peak threshold: 100 count; mass defect: 100 mmu/100 u; background mass: 50 u.

SPE extractions were performed on the Visiprep DL Vacuum manifold for 12 samples (Cat no: 57044) from Supelco (Bellefonte, PA, USA).

Extracts were dried on a Büchi Rotavapor R-200 by means of Büchi Vacuum pump, V-700, both from Büchi (Flawil, Switzerland).

2.2. Materials and reagents

All were of analytical reagent grade. Pyridine, and hydroxylamine·HCl were from Reanal (Budapest, Hungary). Hexane, methanol, ethyl acetate, hexamethyldisilazane (HMDS), bis-(trimethyl-silyl) trifluoroacetamide (BSTFA), *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), *N*-methyl-*N*-*tert*-butyldimethylsilyl-trifluoroacetamide (MTBSTFA), trifluoroacetic acid (TFA) and model compounds such as, androsterone (5α-androstan-3α-ol-17-one), β-estradiol (estra-1,3,5(10)-triene-3,17β-diol), transdehydroandrosterone (androst-5-en-3β-ol-17-one), trans-androsterone (5α-androstan-3β-ol-17-one), mestranol (3-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yn-17-ol), dihydrotestosterone (5α-androstan-3-one-17β-ol), ethinylestradiol (19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol), testosterone (androst-4-en-3-one-17β-ol), norethisterone (19-nor-17α-pregna-4-en-20-yne-3-one-17β-ol), estriol (estra-1,3,5(10)-triene-3,16,17-triol), 4-androstene-3,17-dione (androst-4-en-3,17-dione), gestodene (18a-homo-19-nor-17α-pregna-4,15-dien-20-yne-3-one-17β-ol), levonorgestrel (18a-homo-19-nor-17α-pregna-4-en-20-yne-3-one-17β-ol), etonogestrel (11,18a-dihomo-19-nor-17α-pregna-4,11a-dien-20-yne-3-one-17β-ol), progesterone (pregn-4-en-3,20-dione), coprostanol (5β-cholestan-3β-ol), cholesterol (cholest-5-en-3β-ol), medroxyprogesterone acetate {(6a-homo-pregn-4-en-17α-ol-3-one)-acetate}, stigmasterol (stigmast-5,22-dien-3β-ol) and β-sitosterol (stigmast-5-en-3β-ol) were all from Sigma (St. Louis, MO, USA). Glass microfiber 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).

2.3. Sample preparation for pollutants' GC–MS determinations

2.3.1. Solid phase extraction

Cartridges, prior to extractions were treated with 5 mL hexane, 5 mL ethyl acetate, 10 mL methanol and 10 mL distilled water. Before the SPE enrichment, wastewater samples were filtered on glass microfiber paper (Glass microfiber filters (FF/A 125 mm, ∅, Cat no: 1820-125) which was from Whatman (Maidstone, UK). Car-