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On-line solid-phase extraction coupled to liquid chromatography tandem mass spectrometry optimized for the analysis of steroid hormones in urban wastewaters

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ABSTRACT

An analytical method based on on-line SPE–LC–APCI–MS/MS has been developed for the detection and quantification of eight selected estrogenic and progestagenic steroid hormones; estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethinylestradiol (EE2), levonorgestrel (LEVO), medroxyprogesterone (MEDRO), norethindrone (NORE) and progesterone (PROG) in wastewater matrices. The injection volume could range from 1 to 10-mL according to the expected concentration of steroid hormones in matrix. The method characteristics are: analysis time per sample (<15 min), acceptable recovery values (71–95%), good precision (RSD≤10%) and limits of detection at the low-nanogram per liter levels in affluent and effluent wastewaters (8–60 ng L^{-1}). In particular, a detailed discussion of optimization parameters impacting overall performance of the method has been presented (sample collection, filtration and storage). All optimization and validation experiments for the on-line SPE method and chromatographic separation were performed in environmentally-relevant wastewater matrices. This method represents a compromise between analysis time, higher sample throughput capabilities, sample volume and simplicity for the analysis of both progestagenic and estrogenic steroid hormones in a single run, with LODs and LOQs sufficiently low to detect and quantify them in environmental wastewater matrices. Thus, the applicability of the method was tested on affluent and effluent wastewaters from two wastewater treatment facilities using different processes (biological and physico-chemical) to evaluate their removal efficiency for the detected steroid hormones.

1. Introduction

The monitoring of endocrine disrupting compounds (EDCs), such as steroid hormones, in the aquatic environment is progressively becoming a priority for government agencies, regulatory agencies as well as the general public. They originate from naturally-occurring (e.g. normal urine excretion from mammals) and synthetic sources (e.g. oral contraceptives and hormone replacement therapy). Given their strong endocrine-disrupting potency and their occurrence, selected estrogens, progestagens and androgens have been targeted and detected in wastewater, surface water and drinking water [1–6]. With growing populations and increased discharges from wastewater treatment plants (WWTPs), the presence of steroid hormones in surface waters could be cause for concern since conventional treatment methods have proven to be inadequate to sufficiently eliminate them. There is strong evidence that impacts on the reproductive physiology of wildlife populations occur at very low concentrations, i.e. from 0.1 to 1.0 ng L^{-1} [7–10]. Several studies conducted in numerous countries [2,11], have shown that WWTP effluents and receiving water bodies contain sufficient amounts of estrogenic and progestagenic compounds to induce harmful effects on fish, with their concentrations varying from sub-ng L^{-1} levels to hundreds of ng L^{-1} in wastewater samples [11–13]. Therefore, the development of analytical methods able to detect and quantify these steroid hormones is of critical importance.

To date, numerous analytical procedures have been developed to identify and quantitate steroid estrogenic hormones in water matrixes and often include the use of chromatography (liquid or gas) coupled to tandem mass spectrometry (MS/MS) [5,14]. However, gas chromatographic (GC) methods often require labor and time-consuming steps that improve sensitivity, given the low-molecular weight and low volatility of steroid hormones, but these manipulations could induce some loss of analyte. The necessary sample preparation could include complex hydrolysis as well as derivatization reactions [3,15]. This has led to the development of

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liquid chromatographic (LC) methods that do not necessitate the use of such sample chemical pre-treatment methods for steroid hormones before sample detection, since analyte volatility and thermolability are not limiting factors. Indeed, a review of the literature (from 1981 to the present, i.e. 64 articles) on steroid hormones [3,5,14,16] shows that of the methods developed, 70% used LC, 25% use GC while the remaining 5% used an immunoassay analysis procedure. Pre-concentration and purifying processes, such as solid-phase extraction (SPE) or liquid–liquid extraction (LLE) are necessary because of matrix complexity and the low-nanogram per liter levels at which steroid hormones have been reported in the aquatic environment [3]. More than half (56%) of all LC methods developed used an off-line SPE sample enrichment process that necessitates the use of 100 to 2000 mL of sample volume, making it a slow, tedious and labor-intensive practice. As a result, the identification and quantification of steroid hormones can be time consuming, costly and often result in slow turnover and parsimonious environmental sampling strategies. The concept of on-line SPE where all the steps (conditioning, sample enrichment, wash and elution) involved in the off-line method are integrated into an automated procedure was first introduced in the mid 1990s [17]. This on-line SPE approach, is quicker, allows for reduced sample size, handling and preparation, improved reproducibility, higher sample throughput as well as less waste and solvent consumption.

Only two articles [18,19] have developed an on-line method by LC-MS/MS that include both estrogens and progestagens in the same analytical run, with the use of a 1 to 5 mL injection method with limits of detection (LODs) ranging from 0.3 to 50 ng L^{-1}. The others [16,20–24] have focused on estrogens (including androgens and other analytes of interests) using injection volumes between 1 and 500 mL with LODs between 0.01 and 6.8 ng L^{-1}. Analysis time varies significantly for the proposed methods, with the longest between 25 and 65 min and the others ranging from 10 to 17 min. Also, method validation parameters have not always been clearly defined. Indeed in one study [19], the LODs and limits of quantifications (LOQs) were determined in Milli-*Q* water with the measured concentrations in wastewater samples for estrogens being lower than the reported linearity range. In another work [18], the LODs were determined in river water while the calculated concentrations were reported in wastewater or surface matrices and the LOQ values were not reported for the steroid hormones analyzed. Finally, sample collection, storage and pre-treatment are not discussed in the majority of the methods and could have a significant impact on the reported results.

Our objectives in this study is to optimize and apply an on-line tandem SPE–LC–MS/MS method for the determination of eight selected hormones in water, i.e. estrogens (estriol, estradiol, estrone and 17-α-ethinylestradiol) and progestagens (progesterone, levonorgestrel, medroxyprogesterone and norethindrone). We aim to validate the method starting with sample collection, filtration and storage, up to the on-line pre-concentration followed by LC–MS/MS detection. A proposed method for the determination of LODs and LOQs according to the product ions will be used in order to consider both SRM transitions for the quantification and detection of selected steroid hormones. Their determination at low-nanogram per liter levels in affluent and effluent wastewater was done to confirm the applicability of the method in real environmental samples.

2. Experimental

2.1. Chemicals, reagents and stock solutions

All selected steroid hormone standards (purity≥97%); estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethinylestradiol (EE2), levonorgestrel (LEVO), medroxyprogesterone (MEDRO), norethindrone (NORE) and progesterone (PROG) were purchased form Sigma Aldrich (St. Louis, MO) and are illustrated in Fig. S1. Isotopically-labeled estradiol, [$^{13}C_2$]-E2 was obtained from Cambridge Isotope Laboratories (Andover, MA) and used as internal standard (IS). Individual stock solutions were prepared in methanol (MeOH) at a concentration of 1000 mg L^{-1} and kept at −20 °C for a maximum of six months. A primary mix of steroid hormone working solution was prepared weekly at a concentration of 50 mg L^{-1} by dilution in MeOH of individual stock solutions aliquots. Subsequent working solutions were prepared daily in water to give solutions of desired concentration. All organic solvents and water used for dilutions were of HPLC grade purity from Fisher Scientific (Whitby, ON, Canada).

Analyte-free effluent wastewater samples were generated by maintaining previously collected samples in the laboratory under conditions to favorable to degradation (exposed to light and keep at room temperature) for long periods of time, until the targeted analytes were no longer detectable.

2.2. Instrumental conditions

The pre-concentration of selected steroid hormone water samples was performed using the Equan™ (Thermo Fisher Scientific, Waltham, MA) system. It consists of a sample delivery system, a dual switching-column array and an LC–MS/MS system. The delivery system comprised an HTC thermopal autosampler manufactured by CTC analytics AG (Zwingen, Switzeraland) used for in-loop sample injection and a quaternary pump Accela 600 (Thermo Finnigan, San Jose, CA) used to load the SPE column with the contents of the sample loop. The column switching system was composed of two-position six-port and ten-port valves (VICI® Valco Instruments Co. Inc., Houston, TX) and a quaternary pump Accela 1200 (Thermo Finnigan, San Jose, CA) used for sample elution from the SPE column and separation on the analytical column. The on-line SPE was achieved using two Hypersil Gold aQ (20 mm × 2 mm, 12 μm particle size) columns in tandem and chromatographic separation was done with a Hypersil Gold (100 mm × 2.1 mm, 1.9 μm particle size) column kept at 55 °C. All columns were manufactured by Thermo Fisher Scientific (Thermo Finnigan, San Jose, CA). Ionization of steroid hormones was achieved using the Ion Max API Source mounted on a Quantum Ultra AM triple quadrupole mass spectrometer by Thermo Fisher Scientific (Waltham, MA) operated in selected reaction monitoring (SRM) mode for quantification and detection.

2.2.1. On-line solid phase extraction and chromatographic conditions

In order to improve signal intensities and method detection limits (MDLs) we tested multiple injection volumes of a 150 ng L^{-1} mix steroid hormone solution in HPLC water and affluent wastewater, using a 20-mL injection loop. This allowed us to establish the maximum injectable volume without loss of analyte. According to the optimized procedure, a sample loading volume (using a 5-mL syringe) ranging between 1 and 10 mL is possible and adjustable depending on the expected steroid hormone concentrations in the sample matrix. The sample transfer rate (loading speed) from the injection loop to the SPE column was tested between 1.0 and 5.0 mL min^{-1}, for a concentration of spiked steroid hormones (500 ng L^{-1}) in analyte-free affluent wastewater, in order to reduce total analysis time. The maximum sample loading flow rate from the sample loop (10 mL) to the SPE columns was 1.5 mL min^{-1} from the loading pump using water with 0.1% formic acid (FA). Following the sample loading step, the pre-concentration columns were back-flushed and the eluting analytes were transferred using the analytical pump gradient directly