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Column-switching reversed phase–hydrophilic interaction liquid chromatography/tandem mass spectrometry method for determination of free estrogens and their conjugates in river water

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ABSTRACT

We report a column-switching liquid chromatography (LC) tandem mass spectrometry (MS/MS) method for highly sensitive determination of both free estrogens (estrone, estradiol, and estriol) and their conjugates (estrone-3-sulfate, estradiol-3-sulfate, estriol-3-sulfate, estrone-3-glucuronide, estradiol-3-glucuronide, estriol-16-glucuronide, and estriol-3-glucuronide) in river water. This technique combines reversed phase (RP) chromatographic separation of the dansyl chloride derivatized free estrogens and hydrophilic interaction liquid chromatographic (HILIC) separation of the estrogen conjugates with multiple reaction monitoring (MRM). Using this new method, sensitivity increases 100- to 1000-fold for free estrogens and 2- to 10-fold for estrogen conjugates over RPLC–MS/MS alone. Method detection limits (MDL) range from 0.038 to 6.9 ng L^{-1} with accuracy of 68–105% and precision of 1.7–17%. We successfully used this method to analyze river water samples collected from the North Saskatchewan River at the same location and detected trace concentrations of estrone (0.042 ng L^{-1}) and estrone-3-sulfate (0.84 ng L^{-1}), demonstrating the application of this method for environmental analysis.

1. Introduction

Estrogens are a group of strong endocrine-disrupting chemicals that can cause the feminization of male fish at trace level concentrations (ng L^{-1} range) in aquatic environments [1]. Natural estrogens are excreted by animals and humans mainly as sulfate and glucuronide conjugates in urine, while a small percentage of free estrogen is excreted in the feces [2]. The sulfate and glucuronide conjugates are biologically inactive compared to the free estrogens. However, the conjugates can be transformed to their corresponding free forms by enzymatic hydrolysis or chemical degradation. Therefore, conjugated estrogens are a potential source of free estrogens and determination of both free and conjugated estrogens in environmental samples is important for evaluation of their occurrence, transformation, environmental exposure, and risk assessment. Because of their trace concentrations, similar structures, and complex sample matrices, analysis of these compounds requires high sensitivity and selectivity.

Immunoassays have been useful for the analysis of estrogens in environmental samples [3–5]. These assays are based on antibody binding reactions and are generally sensitive

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and specific. However, only a few antibodies are available for analysis of free estrogens, and cross-reactions are still a problem. Gas chromatography–mass spectrometry (GC–MS) or gas chromatography–tandem mass spectrometry (GC–MS/MS) methods have also been developed [6–11]. GC–MS analysis of estrogens requires derivatization because they are polar and have low volatility. Analysis of estrogen conjugates requires additional chemical or enzymatic hydrolysis before derivatization [6,9], and the concentrations of conjugates are determined by the difference between the concentrations of free estrogens with and without hydrolysis. These methods are time consuming, and incomplete hydrolysis and derivatization reactions can result in poor recovery and inaccurate quantification. Furthermore, the qualitative information needed to identify the conjugates, such as the type of substitution group and its position, is lost during hydrolysis.

High-performance liquid chromatography (HPLC) allows direct analysis of both free estrogens and their conjugates and provides high sensitivity and specificity when in combination with MS or MS/MS detection. Reversed phase (RP) LC–MS (/MS) methods have been used to analyze some free estrogens and estrogen conjugates in water samples [12–19] and river sediments [20]. RPLC separation of estrogen sulfates and glucuronides requires a mobile phase with high aqueous content because of their hydrophilicity, but this does not provide the best conditions for electrospray ionization (ESI) and limits the detection sensitivity of these methods. Our previous study [21] found that hydrophilic interaction liquid chromatography (HILIC) can separate estrogen conjugates using a high organic content mobile phase, which resulted in enhanced sensitivity for MS detection compared to the commonly used RP separation. Unlike the estrogen conjugates, free estrogens lack highly ionizable functional groups (the pKa of estrone, estradiol, and estriol are 10.3, 10.5, and 10.4, respectively [22]), which limits their ionization in solution and thus limits their sensitivity in ESI. Derivatization of free estrogens with dansyl chloride has been proven to greatly increase their ionization efficiency and detection sensitivity [23–26].

In this study, we aim to integrate the advantages of RPLC and HILIC separation to provide highly sensitive and selective analysis of both free estrogens and their conjugates. Our strategy is to derivatize free estrogens with dansyl chloride and separate them with RPLC, and then switch to HILIC for separation of the estrogen conjugates, followed by tandem MS determination. This integrated method will provide enhanced sensitivity for determining free and conjugated estrogens, compared to the RPLC–MS/MS methods. We will demonstrate the capability of this method through the analysis of river water samples.

2. Experimental

2.1. Materials

Dansyl chloride, estrone (E1), estradiol (E2), estriol (E3), estriol-16-glucuronide (E3-16G), sodium salt of estrone-3-sulfate (E1-3S), estradiol-3-sulfate (E2-3S), estrone-3-glucuronide (E1-3G), and estradiol-3-glucuronide (E2-3G) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium salts of estriol-3-sulfate (E3-3S) and estriol-3-glucuronide (E3-3G) were obtained from Steraloids (Newport, RI, USA). Estradiol-2,4,16,16-d_4, sodium salt of estrone-2,4,16,16-d_4 sulfate (E1-3S-d_4), and estradiol-2,4,16,16-d_4 sulfate (E2-3S-d_4) were purchased from CDN Isotopes (Pointe-Claire, QC, Canada). Optima water and acetonitrile (ACN), HPLC grade methanol, analytical grade ethyl acetate, ammonium acetate, sodium bicarbonate, and sulfuric acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Dansyl chloride derivatization of free estrogens

Free estrogens, either dissolved in methanol as standards or in ethyl acetate as extracts from solid phase extraction (SPE), were dried under a stream of nitrogen at 40 °C. The dried residue was re-dissolved in 100 μL of sodium bicarbonate buffer (0.1 M, pH 10) by vortexing for 1 min. This solution was mixed with 100 μL of dansyl chloride in acetone (1 mg mL^{-1}) and vortexed for 1 min. The derivatization reaction was allowed to proceed for 5 min at 60 °C after which the mixture was diluted with 0.8 mL water. The mixture was then loaded onto a Waters Oasis HLB SPE cartridge (30 mg packing material per cartridge; Milford, MA, USA) which had been preconditioned with 1 mL each of methanol and water. After the reaction mixture had run through, the cartridge was washed with 1 mL of water and 1 mL of 50% aqueous methanol to remove the salt and derivatizing reagent. The derivatized estrogens retained on the cartridge were eluted with 1.5 mL of methanol.

2.3. Solid-phase extraction of river water samples

Water samples were collected from the North Saskatchewan River, Edmonton, Canada, at the same location in March 2008. The samples were acidified to pH 2.0 with sulfuric acid and stored in amber glass bottles at 4 °C [18]. Known amounts of labeled surrogates E2-3S-d_4 and E2-d_4 (typically at 4.5 and 1.5 ng L^{-1}, respectively) were spiked into 500 mL aliquots of river water samples. Waters Oasis HLB SPE cartridges (200 mg per cartridge) were conditioned with 6 mL ethyl acetate, 6 mL methanol, and then 6 mL optima water. Each river water sample was passed through the cartridge at 6–8 mL min^{-1} using vacuum. After sample loading, the cartridge was washed with 6 mL of water and dried for 2 h under vacuum. The free estrogens retained on the cartridge were eluted with 6 mL of ethyl acetate, while the estrogen conjugates were eluted with 6 mL of methanol containing 2% ammonium hydroxide. The free estrogen fraction in the ethyl acetate was dried and derivatized with dansyl chloride, and then subjected to SPE as described in Section 2.2. The derivatized estrogens in methanol were mixed with the estrogen conjugates fraction. The mixture was then evaporated to dryness, and the residue was re-dissolved in 100 μL of 90% aqueous ACN containing known amounts of internal standard E1-3S-d_4 (10 ng mL^{-1}).

2.4. Instrumentation

An Agilent 1100 series LC system equipped with a binary pump and an autosampler (Agilent, Waldbronn, Germany) was used for HPLC separations. The single column reversed