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Short communication

Improved method for analyzing estrogens in water by liquid chromatography–electrospray mass spectrometry

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Abstract

An improved LC–electrospray ionization MS method was established for four estrogens (17β-estradiol (E2), estriol (E3), estrone (E1), and ethynyl estradiol (EE)) in environmental water. Almost complete separation of all estrogens was achieved on a phenyl column with methanol/water as the mobile phase. Quantification was achieved in the negative ionization mode using selected ion monitoring. The instrumental detection limits were 20–30 ng/l for the four analytes. In Milli-Q spiked water, the recoveries of the four estrogens were 72–81%, which was similar to those found for river water spiked with the corresponding deuterated estrogens. The detection limits for the four estrogens in river water were 0.1–0.2 ng/l. The method was used to detect residual estrogens in the Tonghui River, which receives water from a municipal sewage treatment plant in Beijing; E1 (1.1 ng/l) and E2 (0.2 ng/l) were detected.

Keywords: Estrogens; LC–ESI-MS; Phenyl column; Environmental analysis

1. Introduction

Natural and synthetic hormones such as 17β-estradiol (E2), estriol (E3), estrone (E1), and ethynyl estradiol (EE) are extremely potent estrogen receptor modulators, and it has been reported that fish exposed to hormones exhibit changes in biomarkers for estrogenicity at concentrations as low as 0.1 ng/l [1–4]. To assess the ecological risk of these compounds, the need for sensitive identification of estrogens in environmental water has increased.

Liquid chromatography–electrospray mass spectrometry (LC–ESI-MS) in the negative ionization mode combined with diverse extraction procedures and elution protocols is increasingly used to quantify estrogens in surface, drinking and sewage treatment water [5–7]. However, it is not easy to analyze estrogens at physiologically active concentrations in environmental water due to the complexity of the environmental matrices and the usually extremely low concentrations of the target compounds. Analyte detectability is greatly improved by eliminating coextraction interferences with immunoaffinity extraction using monoclonal antibodies; however, immunosorbents with long-term stability are not yet available [6]. In addition, instrumental sensitivity should be improved. It is well-known that in LC–MS, the mobile phase composition, i.e., the type of organic solvents and the additives used, can have a significant influence on ionization efficiency in the ESI ion source [5,8,9]. In nearly all LC–ESI-MS methods published to date, acetonitrile was the organic modifier and C18 columns were used to separate the estrogens.

In this paper, we developed a method for the sensitive detection of four estrogens in water, where chromatographic separation was carried out on a phenyl-column with

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methanol/water as the mobile phase. The river water samples were prepared by using C18 SPE combined with a clean-up on Florisil followed by NH_2-SPE.

2. Experimental

2.1. Reagents and materials

Ethinylestradiol (EE), 17β-estradiol (E2), estriol (E3), estrone (E1), d4-E2, d4-E1, and d4-EE were purchased as powders from Wako. Stock standard solutions for each of the analytes were prepared at 1 g/l in methanol. Working solutions of the individual standards and of mixtures of all of them were prepared at various concentrations by appropriate dilution of the stock solutions in methanol. LC-grade solvents acetonitrile and methanol were purchased from Fisher Chemical (China). Ultra pure water was prepared using an Easypure UV Compact Ultrapure System (Fisher Chemical Co., China) under a conductivity of 18.3 $\Omega\,cm^{-1}$. Waters Sep-Pak C18 (1 g, USA), Florisil (1 g, USA) and NH_2 (500 mg, USA) solid-phase extraction cartridges were purchased from the Waters (USA).

2.2. Sample collection

Water samples from the Tonghui River, which receives water from a municipal sewage treatment plant in Beijing, were taken in March 2004. The samples were collected and placed in precleaned glass bottles, and 1% of formaldehyde was added to prevent microbial degradation.

2.3. Sample preparation

The C18 cartridges were conditioned with 6 ml methanol and 6 ml distilled water. Then, 2-l water samples were extracted at a flow rate of 5–10 ml/min. After the cartridges were washed with 10 ml of distilled water, they were dried under a flow of nitrogen for an hour. The analytes were eluted with 6 ml of ethyl acetate–methanol (5:1, v/v) at a flow rate of 1 ml/min. The eluates were dried under a gentle stream of nitrogen. The dry residues were redissolved with 1 ml hexane–methylene chloride (DCM) (1:1, v/v), and passed through the preconditioned Florisil cartridges at a flow rate of 1 ml/min. Ten milliliters of a mixture of hexane–DCM (1:1, v/v) were discarded, and the fraction (F1) containing E1, E2 and EE was eluted with 6 ml of acetone–DCM (1:9, v/v). After the cartridges were rinsed with 6 ml of hexane–ethyl acetate (1:9, v/v), the polar steroid, E3, was eluted with 6 ml water saturated with ethyl acetate, and combined with F1. The solution was evaporated to dryness under a gentle stream of nitrogen, and redissolved with 1 ml methanol, and then passed through an NH_2-SPE cartridge. The filtered solution was collected, and then 5 ml of methanol was passed through the NH_2-SPE cartridge and combined with the filtered solution. The above solution was dried to 0.5 ml under a stream of nitrogen.

2.4. Liquid chromatography

LC was performed on an Alliance 2690 LC (Waters, USA) equipped with a quaternary gradient pump, and an autosampler with a 100 μl injection loop. The injection volume was 20 μl, and the flow rate was kept at 200 μl/min. Capcell Pak C18 (150 mm × 2.0 mm ID, 3 μm, Shiseido, Japan), Xterra MS Phenyl (150 mm × 2.1 mm ID, 3.5 μm, Waters), and UG 120 Capcell Phenyl (250 mm × 2.0 mm ID, 5 μm, Shiseido) were used to separate the four estrogens. When the C18 column was used, the solvent composition gradient was extended from 0% to 100% acetonitrile within 25 min. For Xterra MS Phenyl, the percentage of methanol was linearly increased from 20% to 60% between 0.2 min and 10.2 min after a short isocratic period of 20% methanol, and then linearly increased up to 78% at 28.1 min. For UG 120 Capcell Phenyl, the percentage of methanol was linearly increased from 5% to 55% between 0.2 min and 10.2 min, and then linearly increased up to 100% at 37 min.

2.5. Mass spectrometry

A platform ZMD single quadrupole mass spectrometer (Micromass, Manchester, UK) was used with a Z-Spray ion source fitted with a pneumatically assisted electro-spray probe. The orthogonal Z-Spray interface allowed the entire column effluent from the LC system to be directed into the source without flow splitting, and contributed to the greatly enhanced sensitivity. In the negative mode, typical ion source parameters were used as follows: ESI capillary voltage at 2.5 kV; extractor voltage at 5 V; source block temperature at 130 °C; desolvation temperature at 400 °C; ion energy at 0.8 V; multiplier voltage at 650 V. Nitrogen was used as desolvation gas with a flow rate of about 500 l/h and cone gas with a rate of 100 l/h; the cone voltage was ramped from 50 V to 100 V with the full scan mass ranging from 50 da to 300 da with a scan time of 1.2 s.

3. Results and discussion

3.1. Effects of mobile phase on the sensitivity and separation

The 10-μl standard samples of 1 mg/l for each estrogen were analyzing through flow-injection, and the effects of mobile phase on sensitivity were investigated. It was found that the mobile phase with methanol as an organic modifier produced a higher response in comparison with that using acetonitrile, which is different from the results in ionspray LC–MS [5].

Several C18 columns using acetonitrile as organic modifier have been used to separate the four estrogens. The in-

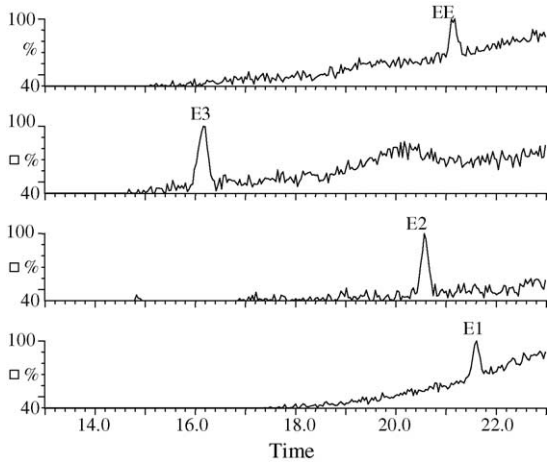

Fig. 1. Extracted LC–MS chromatograms of standard sample for each estrogen at 100 ng/l. Separation conditions: Xterra MS Phenyl column (Waters). Column temperature: 30 °C. Selected ions: *m/z* 269, 271, 287 and 295 for E1, E2, E3 and EE, respectively.

strumental detection limits with LC–ESI-MS were 100, 250, 50 and 500 ng/l at signal-to-noise ratios (S/N) of 3 for E1, E2, E3 and EE, respectively [7], which was similar to those estimated in this study. The sensitivity was improved when using methanol as an organic modifier and the limits of detection of E1, E2, E3, and EE were 50 ng/l, 25 ng/l, 50 ng/l, and 50 ng/l, respectively, which were experimentally estimated from the injection of standard solutions serially diluted until S/N reached a value of 3. Unfortunately, the co-eluant of E1, E2 and EE were found in the chromatogram even when the gradient condition was optimized.

3.2. Improvement of separation and sensitivity for analyzing four estrogens

To achieve a method with both higher sensitivity and separation efficiency, a phenyl column (Xterra MS) combined with the mobile phase of methanol/water was attempted. Fig. 1 shows the chromatograms of a standard mixture containing 100 ng/l for each analyte. There were four distinguishable peaks with S/N ratios of 10–16, and the separation of the four estrogens was greatly improved. It should be noted that the efficiency of chromatographic separation between EE and E1 at 40 °C was found to be lower than that 30 °C. Incidentally, four estrogens were also separated successfully by the UG 120 Capcell Phenyl column. With further dilution of the working standard solution, the detection limits were estimated as follows: 30 ng/l for E1; 20 ng/l for E2; 20 ng/l for E3 and 30 ng/l for EE, which is 2.5–16 times lower than those obtained using CH_3CN/water as a mobile phase [9]. Thus, a method with higher sensitivity and separation efficiency was developed.

3.3. Sample preparation

Recoveries of each step of the sample preparation were evaluated individually. The results are presented in Table 1.

Table 1
Average recoveries of each sample preparation step for E1, E2, E3 and EE

	Recovery (%)			
	E1	E2	E3	EE
C18	86 ± 8	88 ± 9	79 ± 8	84 ± 10
C18 + NH_2	82 ± 4	82 ± 4	78 ± 9	80 ± 7
C18 + NH_2 + florisil	78 ± 8	81 ± 6	72 ± 8	75 ± 4

Two liters of Milli-Q water spiked with 2 ng/l for each estrogen was extracted by C18 SPE combined with a clean-up on Florisil followed by NH_2-SPE. The final recoveries for E1, E2, E3, and EE ranged from 72% to 81%.

3.4. Recovery and detection limit in river water

The extract from a 2 l river water sample spiked with four estrogens at levels of 0.5 ng/l for each estrogen was analyzed, and the SIM chromatograms of EE are shown in Fig. 2. This chromatogram reveals that while a distinguishable peak of EE was found when using the method developed in this study, there was no detectable signal in the chromatogram obtained even for the identical sample by the method based on C18 column separation with CH_3CN/water. The mean recovery of d4-E2, d4-E1, and d4-EE was in the range of 75–81% ($n = 3$), and the limits of detection of E1, E2, E3 and EE in river water were estimated to be 0.1 ng/l for E1, E2 and EE, and 0.2 ng/l for E3. The above result suggests the method established in this study can also improve the sensitivity for analyzing river water samples.

3.5. Environmental samples

Finally, the LC–ESI-MS method established in this study was applied to analyze the residual estrogens in environmen-

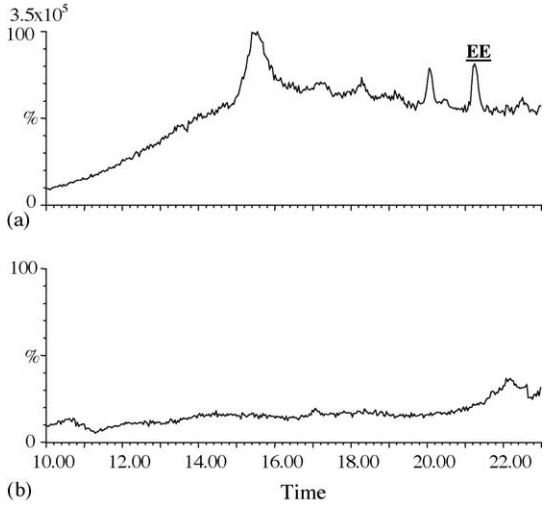

Fig. 2. LC–MS chromatograms of extract in river water spiked by 0.5 ng/l of EE. Separation conditions: (a) Xterra MS Phenyl column and mobile phase: MeOH/H_2O; (b) Capcell Pak C18 column and mobile phase: CH_3CN/H_2O. Selected ion: *m/z* 295.

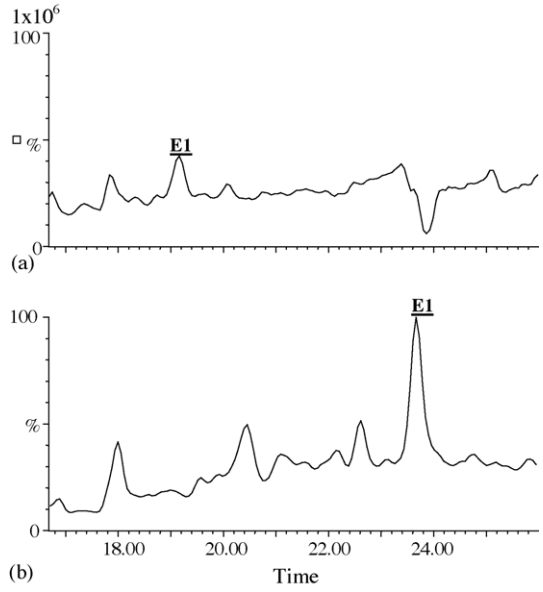

Fig. 3. Extracted LC–MS chromatograms for E1 in river water. (a) C18 column with CH_3CN/water; (b) UG 120 Capcell Phenyl column with CH_3OH/water. Selected ion: *m*/*z* 269.

tal water. Samples taken from the river surface in the Tonghui River, which receives effluent from a municipal sewage treatment plant in Beijing, China, were analyzed to investigate the occurrence of the target components. Fig. 3b shows the chromatogram of the extracts from river water using UG 120 Capcell Phenyl column for separation. Of the four estrogens, only E1 was detected in the water, and the concentration was determined to be 1.1 ng/l. This is consistent with prior reports in the literature for these compounds. E1 is an oxidation product of E2 and may be formed in the river. An identical sample was also analyzed by the method based on C18 combined with CH_3CN/water, and the chromatogram is shown in Fig. 3a. The ratio of signal to noise for the peak corresponding with E1 was 12, which is about 5 times lower than that found in the chromatogram in Fig. 3b, suggesting the effectiveness of the method established in this study.

4. Conclusions

An LC–ESI-MS method with higher sensitivity and separation efficiency was established for analyzing four estrogens in environmental water by adapting a phenyl column with methanol/water for chromatographic separation combined with clean-up on Florisil followed by NH_2-SPE. This technique improved the sensitivity for analyzing four estrogens in river water about several times compared with the method based on C18 separation with a mobile phase of CH_3CN/water.

Acknowledgement

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