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Determination of environmental estrogens in human urine by high performance liquid chromatography after fluorescent derivatization with *p*-nitrobenzoyl chloride

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Abstract

A high performance liquid chromatographic method (HPLC) for the simultaneous determination of 4-nonylphenol, bisphenol A, 17α-ethinylestradiol and three endogenic estrogens including 17α-estradiol, 17β-estradiol, estriol in urine sample, based on precolumn derivatization with *p*-nitrobenzoyl chloride, is presented in this paper. The estrogens mentioned above in urine were firstly hydrolyzed with 0.6 mol/l HCl, and then enriched and cleaned-up by ENV-18 C18 solid phase extraction (SPE) column. The estrogens on column were eluted with dichloromethane, and the eluent was evaporated to dryness under gentle nitrogen flow. The residue was allowed to react with *p*-nitrobenzoyl chloride at 25 °C for 30 min. Separation was performed on a C18 column with gradient elution using acetonitrile and water as mobile phase. A fluorescence detection system was used to detect the fluorescent derivatization products. The detection limit of the method was 2.7 μg/l for bisphenol A and 17β-estradiol, 2.9 μg/l for 4-nonylphenol, 4.6 μg/l for 17α-estradiol and 17α-ethinylestradiol and 8.3 μg/l for estriol, respectively. The relative standard deviations (R.S.D.) ranged from 1.29 to 4.52% and the recoveries ranged from 85.5 to 99.9%. The method was applied to the determination of those six estrogens mentioned above in human urine samples collected from 20 healthy volunteers (aged 21–29). Bisphenol A (BPA) and 4-nonylphenol (NP) were detected with average contents of 1.22 ± 1.38 mg/l and 0.38 ± 0.77 mg/l in 10 male urine samples and 1.29 ± 1.22 mg/l and 0.05 ± 0.05 mg/l in 10 female urine samples, respectively. 17α-ethinylestradiol (α-EE2) was also detected with average contents of 0.13 ± 0.41 mg/l and 0.06 ± 0.15 mg/l in male and female urine samples, respectively.

Keywords: HPLC; Environmental estrogens; Bisphenol A; 4-Nonylphenol; Solid phase extraction (SPE); Precolumn fluorescent derivatization; Human urine; *p*-Nitrobenzoyl chloride; Fluorescence detection

1. Introduction

Chemicals that disrupt endocrine functions have been found in the environment and are linked to adverse effects on the reproductive system in wildlife and human. Many of these chemicals can bind to the estrogen receptors and initiate the estrogen receptor-mediate in vitro. Although environmental exposure to these chemicals has the potential to disrupt reproductive function, the actual impact on the reproductive health has not been defined thoroughly. 4-Nonylphenol is the biodegradation metabolite of the non-ionic surfactants that has been shown to exist in the environment, such as river water and sewage sludge, and also in fish tissue [1–5]. Bisphenol A (BPA) is mainly used in the manufacture of polycarbonate plastics and epoxy resins. It is of great concern that they are used as food and beverage containers and as the lining of metal cans, from which BPA leaches into food [6]. More recently, many reasonable suspicions have been raised concerning the environmental impact of 17α-ethinylestradiol (α-EE2), a synthetic contraceptive chemical as well as the endogenous estrogens, such as 17β-estradiol (β-E2),17α-estradiol (α-E2), estriol(E3) and estrone(E1) excreted by humans and animals that occur in environmental water [7].

The metabolism of estrogens has been investigated both in vivo and in vitro. The results suggested that these estrogens absorbed by the intestine are glucuronidated and sulphated in the liver and kidney and excreted as glucuronide and

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sulphate in the urine. Therefore, urinary estrogens may be a good marker to estimate exposure levels of these estrogens.

In recent years, many novel analytical techniques, such as LC–MS [8], GC–MS [9] and other techniques [10–12] have been developed for the determination of estrogens in water, sediment, tissue and plasma. However, few studies are available for the quantitative analysis of estrogens in human urine. Moreover, most reported methods could only simultaneously determine not more than four estrogens. In liquid chromatographic analysis of estrogens with fluorophores, the detection limits of fluorescence detection are generally one or two orders of magnitude higher than those of UV detection, and quantitation of nanogram to pictogram amount of them is possible, even in complex matrices. But most of estrogens have no native fluorescent properties, so a derivatization reaction is necessary prior to analysis to introduce fluorescent fluorophores into the molecules. Watanabe et al. [13] reported a fluorescence detection method with 4-(4,5-diphenyl-1*H*-imidazol-2-yl) benzoyl chloride for determination of trace levels of BPA in plasma samples and Naassner et al. [14] used Dansyl chloride as a fluorescence label for determination of NP and BPA in sewage sludge. Katayama and Taniguchi [15] developed a method to detect E1, E2, E3 and EE2 in plasma sample using 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole as a fluorescent label. But in these reported methods, some other reagents must be added to accomplish the fluorescence reaction and concomitantly many outgrowths would be produced. *p*-Nitrobenzoyl chloride is a good fluorescent derivatization reagent which can easily react with hydroxyl and phenolic hydroxyl group of an organic chemical without addition other reagents and the reaction condition is also not rigid. In the present study, we employed *p*-nitrobenzoyl chloride as a fluorescent derivatization reagent to introduce fluorophore into the target estrogens molecules, followed by separation with a reverse-phase C18 column and detected with fluorescence detector. The method is applicable to the determination of 4-nonylphenol, bisphenol A, 17α-ethinylestradiol, 17α-estradiol, 17β-estradiol and estriol simultaneously in human urine samples with satisfactory results.

2. Experimental

2.1. Chemicals and reagents

4-Nonylphenol, bisphenol A, 17α-ethinylestradiol, 17α-estradiol, 17β-estradiol and estriol were purchased from Sigma (St. Louis, MO, USA). Estrogens were dissolved respectively in methanol to yield a 1.00 mg/ml stock solution. All of the estrogens were mixed and diluted with methanol to get a working solution containing each estrogen at 10 μg/ml. *p*-Nitrobenzoyl chloride was purchased from Acros Organics (New Jersey, USA) and dissolved in acetonitrile to yield a solution of 0.20 g/ml. (HPLC)-grade acetonitrile was purchased from Tedia (Tedia Company, Inc., USA). All other reagents and solvents were purchased from domestic with analytical reagent grade. Water used in this study was from a Milipore pure water system (Millipore Co., France). Methanol and water were used to activate the solid phase extraction (SPE) columns prior to clean up the sample solution.

2.2. Instrumentation

The HPLC system HP1100 (Agilent Technologies Co. Ltd., USA) was equipped with a fluorescence detector and a workstation for data treatment. The column used was a phenomenex® Luna C18, 5 μm, 250 mm × 4.6 mm (Phenomenex, Torrance, CA, USA) with a phenomenex® guard column, 4 mm length × 3 mm i.d. (Phenomenex, Torrance, CA, USA). A 12-port SPE vacuum manifold (Supelco Visiprep DL, USA), a vacuum pump and 3 ml reversed phase SPE cartridges containing C18 sorbent (ENVI-18, Supelco, 57063) were used to extract and clean up the urine samples.

2.3. Operating conditions

The time program for the wavelength switching of fluorescence detector was that from start to 8.5 min, the excitation wavelength and the emission wavelength were set at 282 and 315 nm, respectively and from 8.5 to 20 min, they were set at 228 and 316 nm, respectively. The chromatographic separation was performed on a column of 250 mm × 4.6 mm (phenomenex®, USA) with a phenomenex® guard column (4.0 mm length × 3 mm i.d.). A mobile phase gradient elution program with solvent A (acetonitrile) and solvent B (water) was applied at a flow-rate of 1.0 ml/min. The gradient program started with 30% of acetonitrile and then increased linearly to 70% of acetonitrile in 12 min. From 12 to 12.3 min, mobile phase A was increased linearly to 100% of acetonitrile and this condition was held until 20 min. From 20 min, the elution program returned to initial condition and held for 20 min. The column temperature was maintained at 25 °C during analysis.

2.4. Sample preparation

Urine samples were collected from 20 health volunteers aged 21–29 (including 10 men and 10 women) at arbitrary time of a day. All samples were stored and frozen at −20 °C until analysis. Estrogens in urine mainly conjugated with glucuronic acid or sulfuric acid, so that the urine samples must be enzymatic or chemical hydrolyzed to release the conjugates before extraction. We adopted chemical hydrolysis because it is much simpler and less time consuming than the enzymatic hydrolysis procedure. We also used SPE columns to extract and clean up the samples for its simplicity, as well as the cartridge only require a small amount of organic solvent [8].

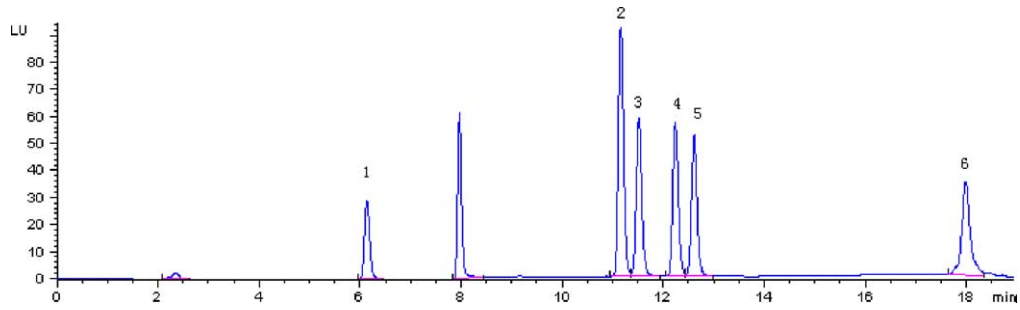

Fig. 1. The chromatogram of standards: (1) estriol, E3: 6.1 min; (2) bisphenol A, BPA: 11.3 min; (3) 17β-estradiol, β-E2: 11.7 min; (4) 17α-estradiol, α-E2: 12.2 min; (5) 17α-ethinylestradiol, EE2: 12.8 min; (6) 4-nonylpheonl, NP: 17.9 min.

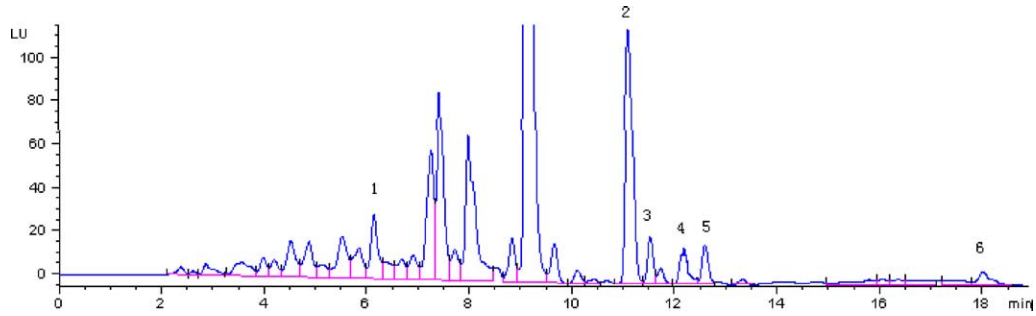

Fig. 2. The chromatogram of a urine sample. The peak identifications are same as that in Fig. 1.

Into 0.50 ml of urine sample, 0.50 ml of methanol and 0.05 ml of concentrated hydrochloric acid were added. And then let the sample stand for 1 h in the incubator of 70 °C for hydrolysis. The hydrolyzed solution was adjusted to pH 3.0 with 5 mol/l sodium hydroxide. SPE cartridges were conditioned prior to use by the sequential addition of methanol (4 ml) and water (4 ml). The cartridges were eluted with dichloromethane (4 ml × 2), and the eluent was evaporated to dryness under gentle nitrogen flow. For recovery experiments, suitable amount of the estrogens was spiked into the urine samples before extraction.

2.5. Derivatization

p-Nitrobenzoyl chloride–acetonitrile solution (0.10 ml of 0.2 g/ml) was added into the sample residue and let it stand for 30 min at ambient temperature (25 °C), then 0.40 ml acetonitrile–water (30:70) was added and vortex agitated for 1 min. The supernatant (20 μl) was injected into the chromatographic system for analysis. To establish a standard curve, a mixture of the target estrogens was subjected to the same procedure as described above; Fig. 1 and Fig. 2 are the chromatograms of standards and urine sample, respectively.

2.6. Quantification

Estrogens were identified by the retention time of the main peak defined by chromatogram of standard estrogens. The estrogen contents were measured through regression equation of estrogen content against its peak area.

3. Results and discussion

3.1. Optimization of the LC–FLD conditions

The chromatographic conditions were carefully studied in order to find a suitable mobile phase with high resolution of the estrogen fluorescent derivatives without quenching of their fluorescence. Mobile phases including methanol and acetonitrile with different eluting programs were tested. Acetonitrile was found more suitable for the purpose and the linear gradient elution could give a satisfactory separation in less than 20 min. Through scanning the excitation wavelength and emission wavelength of E2 and E3 were found to be 282 and 315 nm, respectively, and that for BPA, NP, EE2 were 228 and 316 nm, respectively. It is not easy to switch the wavelength because the peaks of BPA and β-E2, as well as those of α-E2 and EE2, were close to each other. In this study, we focused on the environmental estrogens, whose contents usually lower than those of β-E2, α-E2 and EE2, so we set the excitation wavelength and emission wavelength at 282 and 315 nm, respectively at the beginning, and switched to 228 and 316 nm at 8.5 min even the retention times for β-E2, α-E2 and EE2 were later than 11 min.

3.2. Derivatization conditions

17α-Estradiol, 17β-estradiol and 17α-ethinylestradiol and estriol are non-fluorescence or weak fluorescence estrogens, so they cannot be directly determined by fluorescence detection. We used *p*-nitrobenzoyl chloride as the fluorescent

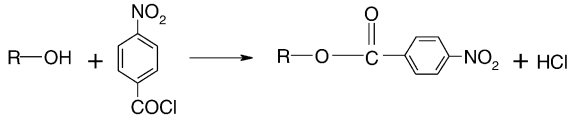

Fig. 3. The fluorescent derivatization reaction.

derivatization reagent for their sensitive analysis. The fluorescent derivatization reaction should proceed under anhydrous condition. The reaction equation is described in Fig. 3. To optimize the reaction conditions, we studied the reaction time and the reaction temperature.

3.2.1. Optimization of the reaction time

We examined the effect of the reaction time on the fluorescence intensity of target estrogens. The results are shown in Fig. 4. From the Fig. 4, we can conclude that between 10 to 30 min, the fluorescence intensity of target estrogens has reached maximum. When the reaction time exceeded 30 min, the fluorescence intensity became weaker gradually. In the study, we fixed the reaction time in 30 min.

3.2.2. Optimization of the reaction temperature

The reaction temperature had a significant influence on the production and the stability of the fluorescent derivatives. From Fig. 5, we can see that the peak area remains maximum when the reaction temperature was at the range of 4–50 °C. In this study, the derivatization reaction was conducted at room temperature (25 °C).

3.3. Optimization of the hydrolysis condition of urine sample

Estrogens are present in biological tissue and fluids mainly as water-soluble conjugates with high polarity, i.e. estrogen sulfates and glucuronides, instead of free compounds. It was reported that estrogens were excreted mainly as glucuronides and the content of estrogen sulfates in human urine was very low. And the content of free estrogens reported was less than 10% [6,8]. This method aimed at detecting the total amount

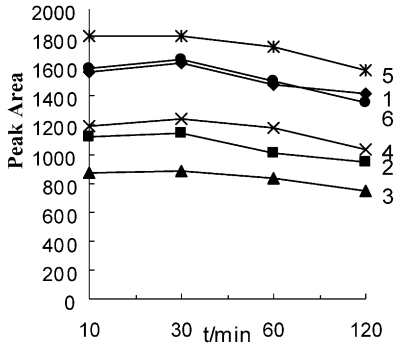

Fig. 4. The influence of derivatization time on peak area: (1) estriol; (2) bisphenol A; (3) 17β-estradiol; (4) 17α-estradiol; (5) 17α-ethinylestradiol; (6) 4-nonylphenol.

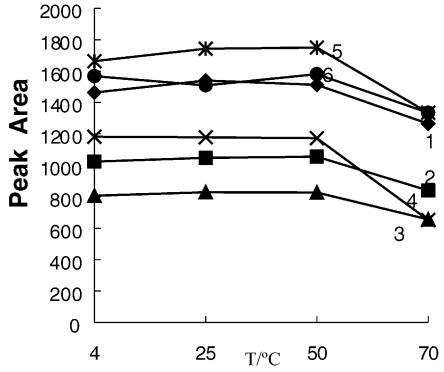

Fig. 5. The influence of derivatization temperature on peak area: (1) estriol; (2) bisphenol A; (3) 17β-estradiol; (4) 17α-estradiol; (5) 17α-ethinylestradiol; (6) 4-nonylphenol.

of estrogens, including the conjugation and deconjugation estrogens. Therefore, the sample must be hydrolyzed prior to analysis. Two methods of hydrolysis have been reported, which involved in enzymatic hydrolysis and acidic hydrolysis. This study selected the latter because it is simple and timesaving.

Under the acidic condition, the conjugated estrogens were not stable, and apt to dissociate to free estrogens. We designed an orthogonal experiment including three factors and four levels. The factors and levels were the concentrations of hydrochloric acid (0.20, 0.60, 1.00, 2.00 mol/l), the temperature of hydrolysis (25, 50, 70, 100 °C), and the hydrolysis time (10, 30, 60, 120 min), respectively. According to the experimental results, we found that the temperature and the concentrations of hydrochloric acid have notable effect on the efficiency of hydrolysis while the effect of the time is not obvious. The hydrolysis efficiency increased until 70 °C and then decreased. The area of peak reached to maximum when the acidity was about 0.6 mol/l. When the hydrolysis time was 60 min, the area of peak would reach to the maximum, and the efficiency of hydrolysis would not be remarkably affected when the hydrolysis time was between 50 and 70 min. In this study, we added 0.05 ml of concentrated hydrochloride acid and 0.50 ml of methanol into 0.50 ml of urine sample, the final concentration of hydrochloride acid in the sample solution was about 0.6 mol/l. Methanol in the sample solution can increase the solubility of the estrogens, so could speed up the hydrolysis process. The temperature and time of hydrolysis we used were 70 °C and 60 min, respectively.

3.4. Method performance

The method performance was evaluated by the determination of the linearity, sensitivity, precision and accuracy of the method. A 20 μl volume of standard mixture of BPA, NP, EE2, E1, β-E2, α-E2 with different concentration was injected into the HPLC system, and calibration curves were constructed by plotting the peak areas against the concen-

Table 1
Calibration curves of E3, BPA, 17β-E2, 17α-E2, 17α-EE2 and NP

Chemical	Equation	Correlation coefficients (r)
Estriol (E3)	$Y = 324.8X + 5.95$	0.9998
Bisphenol A (BPA)	$Y = 938.1X + 31.4$	0.9996
17β-Estradiol (β-E2)	$Y = 681.9X + 19.97$	0.9996
17α-Estradiol (α-E2)	$Y = 642.4X + 16.67$	0.9997
17α-Ethinylestradiol (α-EE2)	$Y = 551.9X + 19.27$	0.9995
4-Nonylphenol (NP)	$Y = 714.8X + 21.81$	0.9996

In the equation, X denotes the concentration of estrogens and Y denotes the peak area.

Table 2
The detection limit, repeatability and recoveries of the method

Estrogens	Detection limit (μg/l)	R.S.D. (%)	Recovery (%)
Estriol (E3)	8.3	1.29	94.4
Bisphenol A (BPA)	2.7	3.92	95.9
17β-Estradiol (β-E2)	2.7	3.31	85.5
17α-Estradiol (α-E2)	4.6	2.60	97.6
17α-Ethinylestradiol (α-EE2)	4.6	2.99	99.9
4-Nonylphenol (NP)	2.9	4.52	92.6

Table 3
The contents of six estrogens in 20 urine samples ($\bar{x} \pm s$)

Estrogens	Male (10 cases)			Female (10 cases)		
	Content range (mg/l)	$\bar{x} \pm s$ (mg/l)	Percentage of detection (%)	Content range (mg/l)	$\bar{x} \pm s$ (mg/l)	Percentage of detection (%)
Estriol (E3)	nd[a]–1.81	0.22 ± 0.57	30	nd[a]–2.18	0.77 ± 0.83	90
Bisphenol A (BPA)	nd[b]–3.95	1.22 ± 1.38	60	0.03–3.74	1.29 ± 1.22	100
17β-Estradiol (β-E2)	nd[b]	nd[b]	0	0.01–10.64	2.24 ± 4.30	100
17α-Estradiol (α-E2)	nd[c]	nd[c]	0	nd[c]–0.15	0.04 ± 0.05	70
17α-Ethinylestradiol (α-EE2)	nd[c]–1.30	0.13 ± 0.41	10	nd[c]–0.49	0.06 ± 0.15	20
4-Nonylphenol(NP)	nd[d]–2.26	0.38 ± 0.77	50	nd[d]–0.14	0.05 ± 0.05	70

[a] nd < 8.3 μg/l.
[b] nd < 2.7 μg/l.
[c] nd < 4.6 μg/l.
[d] nd < 2.9 μg/l.

trations for each chemical (0–10.0 μg/ml). Satisfactory linearity was obtained with $r > 0.9991$ for all the target compounds (Table 1).

The detection limits of the target compounds, defined as a signal-to-noise ratio of 2, are listed in the Table 2. And the recoveries of estrogens were determined by spiking to urine different levels of standard mixture (0.40, 0.80 and 1.60 μg/ml). Recoveries of the estrogens ranged from 85.5 to 99.9%. The repeatability of the method was evaluated by the analysis of five replicates of the samples spiked with a standard mixture of the six estrogens (Table 2).

3.5. Application to human urine samples

Twenty healthy volunteers including 10 men and 10 women were enrolled for collecting urine samples in this study. The six estrogens in all the samples were analyzed with the established method. As indicated in Table 3, all six target estrogens have been detected in part or most of the samples. BPA and NP were detected with high percentage in all urine samples of both men and women. 17β-Estradiol, 17α-estradiol and estriol have been detected in 100, 70 and 90% of women urine samples, respectively. 17α-Ethinylestradiol was detected only 10 and 20% in men and women urine samples, respectively. There are few studies on the detection of BPA and NP in the urine samples. Ouchi and Watanabe [16] reported using HPLC-ED to detect the BPA glucuronide in the urine samples and found to be 0.2–19.1 ng/ml (i.e. 0.0002–0.0191 mg/l) in urine of 48 women college students. Inoue et al. [17] employed column-switching LC with an electrospray MS detector coupled with on-line extraction, enzymatic deconjugation and creatinine correction for the detection of NP and 4-*tert*-octylphenol (OP), and found the urinary NP levels in the healthy volunteers varied from <0.3 to 0.96 ng/ml (i.e. <0.0003–0.00096 mg/l). The very high NP contents in the urine samples of our experimental subjects are perhaps their higher level exposure to NP.

4. Conclusion

The method applied *p*-nitrobenzoyl chloride as fluorescent derivatization reagent for the estrogens, combined with SPE column clean-up, HPLC column separation and fluorescence detection, demonstrated to be simple and highly sensitive for the quantitative analysis of 4-nonylphenol, bisphenol A, 17α-ethinylestradiol, estriol, 17α-estradiol and 17β-estradiol in urine samples. The method may provide a useful way to investigate the environmental exposure levels of the estrogens in human.

Acknowledgements

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