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NOTE

Rapid and sensitive analysis of phthalate metabolites, bisphenol A, and endogenous steroid hormones in human urine by mixed-mode solid-phase extraction, dansylation, and ultra-performance liquid chromatography coupled with triple quadrupole mass spectrometry

He-xing Wang · Bin Wang · Ying Zhou · Qing-wu Jiang

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Abstract Steroid hormone levels in human urine are convenient and sensitive indicators for the impact of phthalates and/or bisphenol A (BPA) exposure on the human steroid hormone endocrine system. In this study, a rapid and sensitive method for determination of 14 phthalate metabolites, BPA, and ten endogenous steroid hormones in urine was developed and validated on the basis of ultra-performance liquid chromatography coupled with electrospray ionization triple quadrupole mass spectrometry. The optimized mixed-mode solid phase-extraction separated the weakly acidic or neutral BPA and steroid hormones from acidic phthalate metabolites in urine: the former were determined in positive ion mode with a methanol/water mobile phase containing 10 mM ammonium formate; the latter were determined in negative ion mode with a acetonitrile/water mobile phase containing 0.1 % acetic acid, which significantly alleviated matrix effects for the analysis of BPA and steroid hormones. Dansylation of estrogens and BPA realized simultaneous and sensitive analysis of the endogenous steroid hormones and BPA in a single chromatographic run. The limits of detection were less than 0.84 ng/mL for phthalate metabolites and less than 0.22 ng/mL for endogenous steroid hormones and BPA. This proposed method had satisfactory precision and accuracy, and was successfully applied to the analyses of human urine samples. This method could be valuable when investigating the associations among endocrine-disrupting chemicals, endogenous steroid hormones, and relevant adverse outcomes in epidemiological studies.

Keywords Phthalate metabolites · Bisphenol A · Endogenous steroid hormones · Mixed-mode solid-phase extraction · Liquid chromatography–tandem mass spectrometry · Urine

Introduction

Phthalates and bisphenol A (BPA) are extensively used in the manufacture of common products [1, 2]. Human exposure to them has been shown to be extensive and has been reported to be associated with various adverse health effects [3, 4]. Studies have suggested that phthalates and BPA are suspected endocrine-disrupting chemicals (EDCs), in particular disrupting the steroid hormone endocrine system [5, 6], indicating the need to investigate the associations among these EDCs, endogenous steroid hormones, and relevant adverse outcomes in epidemiological studies.

In previous epidemiological studies on the associations between phthalate or BPA exposure and endogenous steroid hormone levels in the human body, exposure to phthalate or BPA was typically assessed by the respective urinary metabolite concentrations via liquid chromatography (LC)–

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H.-x. Wang · B. Wang · Y. Zhou (✉) · Q.-w. Jiang
Key Laboratory of Public Health Safety of Ministry of Education, School of Public Health, Fudan University, Room 125, Building 8, No. 130, Dongan Road, Xuhui District Shanghai City 200032, China
e-mail: yingchou@fudan.edu.cn

mass spectrometry (MS); however, for endogenous steroid hormones, their blood or serum concentrations were determined by immune assays [7, 8]. This was time-consuming and expensive, with blood or serum being much more difficult to obtain than urine in epidemiological studies.

Endogenous steroid hormones in the human body are metabolized, mainly by glucuronidation in the liver following excretion in urine, similar to phthalates and BPA [9, 10]. Endogenous steroid hormones in urine can therefore expect to be indicators for the disruption of the steroid hormone endocrine system by exposure to phthalates and/or BPA. This similar metabolic pathway provides the possibility of simultaneous determination of phthalate metabolites, BPA, and endogenous steroid hormones in urine. This study aimed to develop and validate a rapid and sensitive analytical method for simultaneous analysis of phthalate metabolites, BPA, and endogenous steroid hormones in urine by ultra-performance LC (UPLC) coupled with triple quadrupole tandem MS (MS/MS). This is, to the best of our knowledge, the first reported analytical method to accomplish this.

Experimental

Reagents and samples

The fourteen phthalate metabolites were mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono[(2-carboxymethyl)hexyl] phthalate (MCMHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-*n*-butyl phthalate (MBP), monoisobutyl phthalate (MiBP), monomethyl phthalate (MMP), monoethyl phthalate (MEP), monocyclohexyl phthalate (MCHP), monobenzyl phthalate (MBzP), monoisononyl phthalate (MiNP), monooctyl phthalate (MOP), and mono(4-hydroxybutyl) phthalate (MHBP). The four estrogens were estriol (E_3), β-estradiol (β-E_2), 2-methoxyestradiol (2-MeOE_2), and estrone (E_1). The two androgens were testosterone (TE) and androstenedione (AED). The two progestogens were progesterone (PGT) and 21-hydroxyprogesterone (21-HPT). The two glucocorticoids were cortisone (COR) and hydrocortisone (HCO). The other standards were BPA, prednisone (PDN), 4-methylumbelliferone glucuronide, and 4-methylumbelliferone (4-MU). The eleven isotopically labeled internal standards were MMP-$^{13}C_4$, MEP-$^{13}C_4$, MBP-$^{13}C_4$, MECPP-$^{13}C_4$, MEHP-$^{13}C_4$, MBzP-$^{13}C_4$, 4-MU-$^{13}C_4$, β-E_2-d_3, BPA-d_{16}, TE-$^{13}C_3$, and PGT-d_9. All standards were purchased from Cambridge Isotope Laboratories (Cambridge, MA, USA), Sigma-Aldrich (St. Louis, MO, USA), or Dr. Ehrenstorfer (Augsburg, Germany).

First morning urine was used in this study. Urine samples were collected from 30 schoolchildren aged 12–15 years in a Shanghai middle school. Pooled urine was prepared by equal-volume mixing of six urine samples from six participants including three males and three females. This pooled urine was used for optimization and validation of the method.

Sample pretreatment

An aliquot (1.0 mL) of urine was spiked with 20 μL of a standard solution containing six isotopically labeled phthalate metabolites and 4-MU-$^{13}C_4$ at a concentration of 3 μg/mL, four isotopically labeled endogenous steroid hormones and BPA-d_{16} at a concentration of 1.6 μg/mL, PDN at a concentration of 30 μg/mL, and 4-methylumbelliferone glucuronide at a concentration of 12 μg/mL. After being vortexed for 30 s, the sample was buffered with 200 μL of 1.0 M ammonium acetate buffer (pH5.0) and 15 μL of β-glucuronidase from *Helix pomatia* (type H-2, Sigma-Aldrich) was added. The mixture was incubated overnight at 37 °C to hydrolyze the analyte conjugates. The addition of 4-methylumbelliferone glucuronide to the urine sample was used to monitor the enzymatic hydrolytic efficiency exerted by β-glucuronidase [11].

Following incubation, the urine sample was loaded onto an Oasis MAX mixed-mode solid-phase extraction (SPE) cartridge (combining reversed-phase and anion-exchange mechanisms, 150 mg/6 mL, Waters, Milford, MA, USA), which was preconditioned with 4 mL of methanol and 4 mL of pure water. The ammonium acetate buffer retained on the cartridge was washed with 4 mL of pure water, and 4 mL of 50 mM sodium dihydrogen phosphate buffer (pH7.5) was used to retain analytes. The analytes were eluted as two separate fractions. Endogenous steroid hormones and BPA were first eluted with 5 mL of methanol after neutral or basic urine impurities had eliminated with 4 mL of 30 % methanol solution. Subsequently, 4 mL of pure water was used to wash off the methanol, followed by 4 mL of 30 % methanol solution containing 1 % formic acid to remove acidic urine impurities. Finally, the phthalate metabolites were recovered with 5 mL of methanol containing 1 % formic acid.

Both fractions were separately concentrated to dryness at 45 °C under a gentle stream of nitrogen. The residue containing phthalate metabolites was reconstituted in 0.5 mL of 30 % acetonitrile solution for UPLC-MS/MS analysis. The residue containing endogenous steroid hormones and BPA was dansylated [12]. Briefly, 100 μL of 100 mM sodium bicarbonate buffer (pH10.0) and 100 μL of 1 mg/mL dansyl chloride solution in acetone were added to the residues. After vortexing for 1 min, the mixture was heated at 60 °C for 10 min, and then cooled to room temperature. The final volume was adjusted to 500 μL with sodium bicarbonate buffer (pH10.0)/acetone (50:50, v/v). The mixture was homogenized for UPLC-MS/MS analysis.

Instrumental analysis

An Acquity UPLC system coupled to a Xevo TQ-S triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Waters, Milford, MA, USA) was used for the measurement of target compounds. Chromatographic separation was achieved using a C_{18} column (100 mm × 2.1 mm × 1.7 μm, Acquity UPLC BEH C_{18}). Two analytical methods were used for phthalate metabolites, and endogenous steroid hormones and BPA. The phthalate metabolites were detected in negative ion mode with a mobile phase of acetonitrile/water containing 0.1 % acetate acid, and endogenous steroid hormones and BPA were detected in positive ion mode with a mobile phase of methanol/water containing 10 mM ammonium formate, both at a flow rate of 0.3 mL/min. For the former, the acetonitrile gradient program was as follows: 0 min (5 %), 1.0 min (25 %), 9.0 min (55 %), 11.0 min (100 %), 12.0 min (100 %), 12.5 min (5 %), and 15.0 min (5 %). For the latter, the methanol gradient program was as follows: 0 min (20 %), 3.0 min (50 %), 6.0 min (80 %), 9.0 min (95 %), 11.0 min (100 %), 11.5 min (20 %), and 14 min (20 %).

In MS, multiple-reaction monitoring mode was used for data acquisition. Capillary voltages were set to 3.0 kV and 2.8 kV for positive ion and negative ion modes, respectively. Other MS settings were identical for both positive ion and negative ion mode: the flow rate of the nebulization gas (nitrogen) was set to 800 L/h at a temperature of 400 °C, the flow rate of the cone gas was set to 40 L/h, and the source temperature was set to120 °C. Argon was used as the collision gas. The capillary energy and the sampling cone voltage were individually optimized by direct syringe infusion of each compound into the mass spectrometer. On the basis of the specificity of the compounds and the sensitivity of the method, multiple ion transitions were selected for each compound (Table 1). One of them was used for quantification, and others were used for confirmation. The quantification ion chromatograms of target compounds in one real urine sample are displayed in Fig. S1. The quantification ions of dansylated estrogens and BPA were identical, and had a mass of 171, and were derived from the derivatization reagent of dansyl chloride.

Quantification

All the target compounds were quantified using internal standard calibration. The calibration curve was based on the peak area of the quantification ion chromatogram for each compound in multiple-reaction monitoring mode. The concentration range of the calibration curve differed by the analyte (Table 2), and each concentration was spiked with the same concentration of internal standards as real urine samples. Calibration standards of endogenous steroid hormones and BPA were derivatized in the same way as real urine samples. The internal standard curve for each target compound was plotted as the ratio of the quantification ion peak area of the target compounds to that of the corresponding internal standard against the mass concentration of the target compound (ng/mL) with $1/x$ weighing. MMP-$^{13}C_4$ was used as the internal standard for MHBP and MMP in the construction of the calibration curve. MEP-$^{13}C_4$ was used for MEP, 4-MU-$^{13}C_4$ was used for 4-MU, MECPP-$^{13}C_4$ was used for MECPP and MEHHP, MBP-$^{13}C_4$ was used for MBP, MiBP, MEOHP, and MCMHP, MBzP-$^{13}C_4$ was used for MBzP and MCHP, MEHP-$^{13}C_4$ was used for MEHP, MOP, and MiNP, β-E_2-d_3 was used for E_3, β-E_2, 2-MeOE_2, and E_1, BPA-d_{16} was used for BPA, TE-$^{13}C_3$ was used for TE and AED, PGT-d_9 was used for PGT and 21-HPT, and PDN was used for COR and HCO.

Results and discussion

Optimization of sample pretreatment

Optimization of SPE

Matrix effects during ESI are caused by coelution of residual components from the sample matrix, which can suppress or enhance ionization of the analytes, resulting in diminished precision and accuracy. The matrix effect was generally calculated as the percent extent of suppression or enhancement of analyte ionization by comparing signal intensities of analytes added to solvent with those added to the sample extracts. It has been reported that the separation of neutral, acidic, or basic compounds in mixed-mode SPE can eliminate the matrix effect caused by each of them, and might be important for analysis of compounds of interest in complex sample matrixes, such as wastewater, urine, and blood [13, 14].

Given the greater convenience of single-mode SPE than mixed-mode SPE, the performance of mixed-mode SPE (reversed-phase and anion-exchange Oasis MAX cartridges) and single-mode SPE (reversed-phase Oasis HLB cartridges) was compared in terms of the matrix effect at a spiking level of 200 ng/mL. Oasis MAX cartridges could separate neutral or weakly acidic endogenous steroid hormones and BPA from acidic phthalate metabolites, but HLB cartridges could not. The protocol for the HLB cartridges was briefly described as follows: after 1.0 mL of pooled urine had been hydrolyzed enzymatically and the pH had been adjusted to 2.0, it was loaded on an HLB cartridge (150 mg/6 mL, Waters), which was preconditioned with 4 mL of methanol and 4 mL of water. Subsequently, the cartridge was washed with 4 mL of 10 % methanol solution

Table 1 Settings for multiple-reaction monitoring

Compound	RT (min)	CV (V)	Precursor ion[a]	Quantification ion[b]	Confirmation ion[b]
21-HPT	6.50	40	331	109 (26)	271 (21), 97 (30)
PGT	7.15	40	315	109 (25)	97 (30), 279 (19)
PGT-d_9	7.12	40	324	113 (25)	100 (30), 288 (19)
COR	4.95	45	361	163 (25)	299 (27), 258 (30)
HCO	5.19	40	363	121 (25)	297 (18), 267 (21)
PDN	4.86	35	359	237 (25)	265 (20), 147 (30)
TE	6.39	40	289	109 (26)	253 (20), 97 (30)
TE-d_3	6.39	40	292	112 (26)	256 (20), 100 (30)
AED	6.14	40	287	109 (26)	97 (30), 211 (21)
E_1	8.71	50	504	171 (33)	440 (29), 425 (38)
E_3	7.91	50	522	171(38)	458 (27), 426 (32)
2-MeOE_2	8.70	50	536	171 (36)	472 (27), 302 (32)
β-E_2	8.83	50	506	171 (42)	427 (34), 491 (28)
β-E_2-d_3	8.83	50	509	171 (42)	430 (34), 494 (28)
BPA	9.77	50	695	171 (50)	461 (37), 368 (42)
BPA-d_{16}	9.73	50	709	171 (50)	411 (42), 475 (37)
MMP	3.05	15	179	77 (17)	107 (15), 121 (15)
MMP-$^{13}C_4$	3.02	15	183	79 (17)	109 (15), 124 (15)
MCHP	7.22	25	247	77 (20)	121 (17), 147 (14)
MEHP	10.81	25	277	134 (16)	233 (10), 147 (16)
MEHP-$^{13}C_4$	10.80	25	381	137 (16)	236 (10), 151 (16)
MOP	11.04	25	277	127 (19)	233 (8), 205 (10)
MBP	6.15	20	221	177 (10)	147 (14), 134 (14)
MBP-$^{13}C_4$	6.15	20	225	180 (10)	151 (14), 124 (14)
MiBP	6.01	20	221	177 (10)	121 (14), 147 (14)
MHBP	3.06	20	237	121 (17)	89 (15),77 (17)
MBzP	6.44	20	255	183 (13)	147 (16), 105 (13)
MBzP-$^{13}C_4$	6.44	20	259	185 (13)	151 (16), 107 (13)
MEHHP	5.92	25	293	121 (20)	145 (14)
MEP	3.74	20	193	77 (13)	121 (15), 147 (13)
MEP-$^{13}C_4$	3.74	20	197	79 (13)	124 (15), 151 (13)
MEOHP	6.08	25	291	121 (18)	143 (15)
MCMHP	6.29	10	307	159 (12)	113 (14)
MECPP	5.58	15	307	159 (12)	121 (12)
MECPP-$^{13}C_4$	5.58	15	311	159 (12)	124 (12)
MiNP	11.06	30	291	141 (21)	219 (17), 247 (14)
4-MU	3.37	15	175	133 (22)	147 (22), 119 (28)
4-MU-$^{13}C_4$	3.35	15	179	135 (22)	150 (22), 121 (28)

RT retention time, *CV* cone voltage, *21-HPT* 21-hydroxyprogesterone, *PGT* progesterone, *COR* cortisone, *HCO* hydrocortisone, *PDN* prednisone, *TE* testosterone, *AED* androstenedione, *E_1* estrone, *E_3* estriol, *2-MeOE_2* 2-methoxyestradiol, *β-E_2* β-estradiol, *BPA* bisphenol A, *MMP* monomethyl phthalate, *MCHP* monocyclohexyl phthalate, *MEHP* mono(2-ethylhexyl) phthalate, *MOP* monooctyl phthalate, *MBP* mono-*n*-butyl phthalate, *MiBP* monoisobutyl phthalate, *MHBP* mono(4-hydroxybutyl) phthalate, *MBzP* monobenzyl phthalate, *MEHHP* mono(2-ethyl-5-hydroxyhexyl) phthalate, *MEP* monoethyl phthalate, *MEOHP* mono(2-ethyl-5-oxohexyl) phthalate, *MCMHP* mono[(2-carboxymethyl)hexyl] phthalate, *MECPP* mono(2-ethyl-5-carboxypentyl) phthalate, *MiNP* monoisononyl phthalate, *4-MU* 4-methylumbelliferone

[a] $[M+H]^+$ for endogenous steroid hormones and BPA, and $[M-H]^-$ for phthalate metabolites

[b] The collision energy (V) is given in *parentheses*.

containing 2 % formic acid and 4 mL of 10 % methanol solution containing 2 % ammonium hydroxide. All analytes were eluted with 5 mL of methanol. The elute was evaporated to dryness, and was dansylated for analysis by LC-MS/MS. The matrix effect for steroid hormones and BPA ranged from 9.9 to 27.4 % in mixed-mode SPE, but from 73.1 to 93.3 % in single-mode SPE. No significant matrix effect differences were observed for analysis of phthalate metabolites. The separation of neutral or weakly acidic endogenous steroid hormones and BPA from acidic phthalate metabolites in reversed-phase and anion-exchange mixed-mode SPE was important to decrease the matrix effect for the analysis of endogenous steroid hormones and BPA.

Following the mixed-mode SPE mechanism, the separation requires pH control to make endogenous steroid hormones and BPA neutral species and phthalate metabolites negatively charged ones. For this, a series of buffer solutions across a pH range of 5–11 were examined by the absolute recovery of each analyte added to pure water. The absolute recoveries of endogenous steroid hormones were similar

Table 2 Linearity, sensitivity, matrix effects, accuracy, and precision of the proposed method

Compound	Linearity		Sensitivity		Matrix effect[a] (%)		Mean recovery (RSD)[b]		
	Range	*r*	LOD (ng/mL)	LOQ (ng/mL)	50	100	Low[c]	Medium[c]	High[c]
21-HPT	0.1-200	0.996	0.05	0.17	26.2	21.5	109.1 (11.2)	97.7 (7.8)	101.0 (6.0)
PGT	0.1-200	0.993	0.04	0.13	28.6	23.8	113.6 (8.5)	101.0 (5.6)	98.7 (6.0)
COR	0.5-200	0.996	0.18	0.59	10.9	8.9	113.5 (14.2)	110.1 (7.5)	96.1 (7.7)
HCO	0.5-200	0.997	0.22	0.73	13.2	10.6	111.5 (12.9)	105.3 (10.4)	96.7 (8.2)
TE	0.1-200	0.995	0.03	0.10	9.9	8.9	104.7 (3.7)	102.9 (2.2)	100.9 (2.7)
AED	0.1-200	0.998	0.05	0.17	8.7	7.5	112.0 (8.9)	105.6 (9.8)	107.6 (5.3)
E_1	0.1-200	0.993	0.03	0.10	9.1	4.7	86.3 (14.6)	101.2 (10.8)	87.1 (8.2)
E_3	0.1-200	0.994	0.03	0.10	10.2	5.2	126.7 (12.1)	113.8 (8.3)	101.9 (6.7)
β-E_2	0.1-200	0.996	0.03	0.10	7.7	3.9	103.8 (14.9)	102.1 (8.8)	95.3 (5.5)
2-MeOE_2	0.1-200	0.994	0.03	0.10	6.1	2.6	100.7 (15.0)	104.9 (6.4)	97.7 (4.3)
BPA	0.1-200	0.992	0.04	0.13	4.5	2.5	76.5 (9.8)	84.7 (4.6)	95.6 (4.0)
MMP	0.5-200	0.996	0.32	1.05	25.1	16.6	109.8 (9.1)	101.5 (7.2)	99.1 (7.2)
MCHP	0.2-200	0.998	0.18	0.58	33.5	20.8	110.6 (10.1)	103.9 (8.1)	93.5 (6.8)
MEHP	0.2-200	0.998	0.11	0.35	25.3	19.4	102.7 (10.6)	116.2 (5.1)	103.0 (5.4)
MOP	0.2-200	0.9993	0.11	0.35	22.8	17.2	113.3 (11.6)	113.6 (5.8)	113.6 (5.8)
MiBP	0.2-200	0.9997	0.32	1.05	21.8	13.7	112.0 (11.0)	100.7 (10.0)	101.1 (7.8)
MBP	0.2-200	0.996	0.35	1.17	22.3	14.9	125.0 (11.0)	114.9 (6.6)	106.5 (4.0)
MHBP	1.0-200	0.9991	0.84	2.80	28.9	19.5	92.9 (22.0)	96.0 (11.0)	93.4 (7.7)
MBzP	0.2-200	0.998	0.14	0.47	35.5	22.3	101.0 (16.3)	98.2 (8.3)	96.0 (3.9)
MEHHP	0.5-200	0.952	0.39	1.28	21.2	15.7	79.4 (12.6)	86.2 (8.3)	91.1 (5.1)
MEP	0.5-200	0.998	0.46	1.52	19.3	23.7	118.5 (12.0)	101.5 (10.7)	104.5 (4.1)
MEOHP	0.5-200	0.996	0.35	1.17	18.9	10.4	119.2 (12.5)	111.2 (5.4)	88.8 (6.4)
MCMHP	0.2-200	0.966	0.21	0.70	20.3	11.2	119.7 (16.9)	112.2 (9.9)	99.9 (4.3)
MECPP	1.0-200	0.996	0.56	1.87	18.7	13.8	111.3 (12.0)	113.4 (7.1)	102.8 (5.5)
MiNP	0.2-200	0.998	0.11	0.35	26.2	20.1	118.8 (13.9)	117.9 (9.3)	111.6 (4.5)

LOD limit of detection, *LOQ* limit of quantification, *RSD* relative standard deviation

[a] Assessed at two different spiking concentrations: 50 and 100 ng/mL

[b] Accuracy and precision are expressed as the mean recovery (%) and the RSD (%), respectively.

[c] For steroid hormones and BPA, low, medium, and high refer to spiking concentrations of 2, 10, and 50 ng/mL, respectively, whereas for phthalate metabolites they refer to 10, 50, and 100 ng/mL.

across the pH range examined as expected. However, the recoveries of BPA began to decrease at pH8, and were 20.3 % and10.8 % at pH9 and 11, respectively. The recoveries of phthalate metabolites were similar across the pH range, with the exception of MECPP, whose absolute recoveries increased with increasing pH, and were 75.3 % and 80.5 % at pH7 and 8, respectively. So, a pH range of 7–8 yielded acceptable recoveries for all analytes. Finally, a 50 mM sodium dihydrogen phosphate buffer of pH7.5 was chosen as optimal.

Dansylation of estrogens and BPA

Dansyl chloride was used to give estrogens and BPA a dimethylamino group, favoring positive ion mode, because they contain a phenolic hydroxyl group. This allowed not only the dansylated estrogens and BPA to be determined together with other endogenous steroid hormones, for which positive ion mode is preferred, in one run, but also increased the analytical sensitivities for the dansylated derivatives [12]. To gain a stable and high derivatization yield for estrogens and BPA, the reaction heating time, pH, temperature, and dansyl chloride concentration were optimized. The optimization was started by heating the sample at 60 °C for 5 min at pH9.0 with a dansyl chloride concentration of 1 mg/mL [15]. When one parameter was being optimized, the other parameters were kept constant. The results showed that estrogens were completely dansylated over heating time range examined from 5 to 30 min. However, for BPA, the dansylation yields increased from 51.2 to 98.7 % when the heating time was increased from 5 to 10 min and similar yields were observed for 10, 20, and 30 min. The dansylation yields for estrogens and BPA

increased with pH in a pH range from 4.5 to 9.5, and were nearly 100 % for a pH between 9.5 and 11.5. No significant dansylation yield differences were observed for the dansyl chloride concentrations (1, 2.5, and 5 mg/mL) or reaction temperatures (60, 70, and 80 °C) examined. Therefore, the final dansylation conditions were set to 60 °C for 10 min at pH 10.0 with a dansyl chloride concentration of 1 mg/mL.

Validation of the method

Matrix effect, linearity, and sensitivity

As shown in Table 2, matrix effects were assessed at two concentrations: 50 and 100 ng/mL. The matrix effect ranged between 2.5 % (BPA) and 28.6 % (PGT) for endogenous steroid hormones and BPA, and between 10.4 % (MEOHP) and 35.5 % (MBzP) for phthalate metabolites at both concentrations. The positive matrix effect suggested that ionization of target compounds in the ESI source was suppressed at different levels. However, the matrix effect could be compensated by the internal standards.

The correlation coefficients of all target compounds were higher than 0.992, except for MEHHP (0.952) and MCMHP (0.966). This showed good linearity for most target compounds over the concentration range of two or three orders of magnitude examined. The limit of detection and the limit of quantification were calculated as a signal-to-noise ratio of 3 and 10, respectively, from the chromatograms of urine samples spiked with the lowest concentration of analytes tested. The limits of detection of endogenous steroid hormones and BPA ranged from 0.03 to 0.22 ng/mL and those of phthalate metabolites ranged from 0.11 to 0.84 ng/mL.

Accuracy and precision

Accuracy and precision were assessed by recovery and relative standard deviation (RSD), respectively. Three sets of six duplicate pooled urine samples containing the same concentrations of internal standards were prepared. For each set, one of six duplicate urine samples was not spiked and was used for determination of the background values of the target compounds, and the other five duplicate urine samples were spiked with analytes at low, medium, and high concentrations of the target compounds. This analytical procedure was repeated on three consecutive days for the assessment of interday accuracy and precision.

Table 2 indicates that the interday mean recoveries and RSDs of endogenous steroid hormones and BPA at the lowest spiking concentration ranged between 76.5 and 126.7 %, with RSDs from 3.7 to 15.0 %. For phthalate metabolites, the interday mean recoveries at the lowest concentration ranged between 79.4 and 125.0 %, with RSDs from 9.1 to 22.0 %.

Application to human samples

The method was successfully applied to analyses of human urine samples from 30 schoolchildren, following the criteria in European Commission Decision 2002/657/EC [16]. Two product ions (one for quantification, the other for confirmation) were simultaneously monitored, and the identification of detected analytes fulfilled the established criteria. Quantification ion chromatograms of analytes in a human urine sample are shown in Fig. S1.

As listed in Table S1, except for 2-MeOE_2, MOP, and MiNP, all analytes were detected in the human urine samples. Urine endogenous steroid hormone concentrations were comparable to those from previous studies [17, 18]. As expected, the TE concentration is higher in boys than in girls, whereas the concentrations of E_1, E_3, and β-E_2 are lower. Moreover, the two glucocorticoids (COR and HCO) were present in higher concentrations than the other steroid hormones. For phthalate metabolites, the urine concentrations fall within the range reported by most previous studies, with the exception of MBzP, which was detected in almost 100 % of study participants in most previous studies, but in only 36.7 % in this study [19–21]. The urine BPA concentration tended to be lower than in most previous studies [22, 23]. However, owing to small sample size in this study, further comparison was not done. These differences may reflect the regional differences in production and use of phthalates or BPA.

Conclusions

We have developed and validated a rapid and sensitive method for simultaneous determination of 14 phthalate metabolites, BPA, and ten endogenous steroid hormones in human urine. Mixed-mode SPE significantly decreased matrix interferences for the analysis of endogenous steroid hormones and BPA. The dansylation of estrogens and BPA shortened the chromatographic separation time and enhanced sensitivities. The data obtained showed this method has satisfactory precision and accuracy. The presence of analytes was confirmed in human urine samples according to the criteria in European Commission Decision 2002/657/EC. This proposed analytical method would be helpful to investigate the associations among EDCs, endogenous steroid hormones, and relevant adverse outcomes in epidemiological studies.

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