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Analysis of environmental biomarkers in urine using an electrochemical detector

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Abstract

Phenols are present in the environment and are prevalent in human populations, as environmental contaminants, dietary components, or their metabolites. Many are potential endocrine-altering agents. Currently available methods analyze single components or single families of chemicals as biomarkers of exposure. In order to assess multiple biologically relevant exposures to such substances, we evaluated the feasibility of determining several phenols simultaneously in urine, using an electrochemical detector (ECD) in combination with high performance liquid chromatography (LC). Based on reported analyses in the literature and the ECD response, we selected four xenobiotic residues, including three phytoestrogens (enterolactone, daidzein, and genistein) and *bis*phenolA [BPA]. These compounds had detection limits below 1 μg/L in urine using the cleanup procedure (glucuronidase hydrolysis and C18 column) and the urine volume (2 mL) we employed. As a pilot study to demonstrate the method's utility, we determined urinary enterolactone, daidzein, genistein and BPA in samples from nine children and 24 adults.

Keywords: Phytoestrogens; *Bis*phenolA; Electrochemical detector; Urine; LC

1. Introduction

Polyphenols and phenolic metabolites of environmental agents have been identified as potential agents for altering endocrine function. In humans, phenolic biomarkers have been determined to evaluate both acute and chronic exposures to such substances [1]. These agents include the phytoestrogens, alkyl phenols, pesticide metabolites, PCBs, and polycyclic aromatic hydrocarbons (PAH). They are derived from the diet, home and personal product additives, pesticides, and air pollution. The observed biomarker determinations in some recent studies suggest that they are among the most prevalent xenobiotic chemicals in the body [2] and possibly the most biologically active [3].

The toxicological importance of such chemicals is recognized, yet exposures are generally characterized singly rather than in ecologically relevant mixtures. The electrochemical detector provides possible means of characterizing multiple phenolic compounds at concentrations found in human urine. Methods using spectrophotometric detection have higher detection limits [4–6], and the highly sensitive and specific mass spectrometric methods [7] are more expensive[8]. Therefore, we evaluated the applicability of an LC-ECD technique to phenolic residues in urine that have been found previously to be common exposures from the the diet or environment.

2. Experimental

2.1. Chemicals

Acetonitrile, methanol (Fisher Scientific, Fair Lawn, NJ, USA), and water (J.T. Baker, Phillipsburg, NJ, USA) were LC grade. Acetate buffer (pH 4.8) was from Electron Microscopy Sciences (Fort Washington, PA, USA) and acetate buffer (pH 4.0) was from Mallinckrodt (Paris, KY, USA). Glucuronidase enzyme, 4-nonylphenol, and ascorbic acid were purchased from Sigma (St Louis,

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MO, USA). Daidzein, genistein, and flavone were obtained from Indofine Chemical Co. (Somerville, NJ, USA). *Bis*phenolA and 4,4′-[2-methylpropylidene] bisphenol were from Wako (Osaka, Japan). Enterolactone was obtained from Fluka (Steinheim, Switzerland). 1-Hydroxypyrene, and pentachlorophenol were from Aldrich (Milwaukee, WI, USA). 3,5,6-Trichloropyridinol and 4-hydroxy-2′,3,4′,5,6′-pentachlorobiphenyl were from Accustandard (New Haven, CT, USA).

2.2. LC conditions

LC analyses were carried out on Star chromatographic system (Varian, Sugarland, TX, USA) with a 9100 autosampler, a 9012 solvent delivery pump, a 9050 UV detector, and a dual channel electrochemical detector (ECD), Coulochem III (ESA Inc., Chelmsford, MA, USA). A data collection system running Varian Star 6.2 software was used for controlling the instrument and collecting the data from the UV detector. Also, we used Turbochrom 6.2 (Perkin-Elmer Inc., Norwalk, CT, USA) to collect data from the UV and electrochemical detectors. A Nova-Pak C18 column (150 mm × 3.9 mm, 4 μm) from Waters (Milford, MA, USA) with ODS guard column (45 mm × 4.6 mm) from Beckman (Fullerton, CA, USA) was used. Mobile phases were A: 50 mM sodium acetate, pH 4.8; B: methanol; C: acetonitrile. The gradient over 45 min was A: 75–10%, B: 23–76%, C: 2–14%, a modification of the method reported by Gamache and Acworth [9]. The flow rate was maintained at 0.7 mL/min, with the column at 35 °C. The ECD detector settings during routine determinations were for oxidation E1 = 100 mV, E2 = 560 mV and for reduction E1 = 550 mV, E2 = 0 mV. The UV detector monitored at 260 nm from 0 to 30 min and 280 nm from 30 to 45 min (a better wavelength for late-eluters). Twenty microliters was injected onto the LC by autosampler.

2.3. Sample preparation and enzymatic hydrolysis

Spot urine samples were obtained in two studies of phytoestrogen exposure, one of 9 year old girls. The other study was a clinical population (10 males, 14 females, median age 52.5 years). Both studies were approved by the Mount Sinai School of Medicine Institutional Review Board. Urine samples were stored in polystyrene tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and ascorbic acid was added (100 mg/100 mL) prior to storage at −20 °C. The sample preparation was modified after that of [5]. Frozen urine samples were thawed, vortex-mixed, and centrifuged at 4000 × *g* for 5 min. Clear supernatant (2 mL) was mixed with 0.5 mL of 0.2 M acetate buffer (pH 4.0), 0.1 mL of 2.8 ppm 4,4′-[2-methylpropylidene] bisphenol (for ECD) and 0.1 mL of 60 ppm flavone (for UV) as internal standards, 50 mg ascorbic acid, and 0.1 mL glucuronidase enzyme. The mixture was incubated at 37 °C with gentle shaking overnight.

The mixture was then applied to a 6 mL C18 disposable extraction column (J.T. Baker, Phillipsburg, NJ, USA) that had previously been conditioned with 5 mL of methanol and 5 mL of acetate buffer (pH 4.0). The column was washed with 2 mL acetate buffer (pH 4.0), and the analytes were eluted with 5 mL methanol. The eluate was dried under a stream of dry nitrogen to less 1 mL and brought up to 1 mL with methanol.

2.4. Environmental contaminants and their metabolites

From the biologic and exposure literature, we selected compounds that had been reported to be prevalent, at high concentrations, or that are biologically active endocrine agents. In order of approximate concentrations in the population, these included the phytoestrogens enterolactone, daidzein, genistein, two alkyl phenols (*bis*phenolA [BPA], nonylphenol), the chlorpyrifos metabolite 2,5,6-trichloropyridinol (TCPy), pentachlorophenol (PCP), and hydroxpyrene (HOPy). We looked in depth at the phytoestrogens, BPA, TCPy, PCP and HOPy.

2.5. Standards and QC

Primary standard stock solutions (100–900 ppm) were prepared by dissolution in ethanol and were stored at −20 °C. Working standard mixtures were prepared by dilution of stock standards in methanol. Three quality control urine pools were created by combining discarded urine (two native pools A and B) and by adding standards to pool A to check recovery. The calibration curves were established with every batch using duplicate injections of nine concentrations of standards from 0.8 to 1000 μg/L; $r^2 \geq 0.990$. Accuracy and precision were determined from quality controls run in each of nine successive batches. For this purpose, one diluted aqueous standard, a water blank, and two duplicates from one of the QC pools were included with every 12 samples for processing. Peak identification of unknown samples was based on relative retention times ([RRT] within 1% of standards) and UV absorption patterns of authentic standards analyzed in the same batch.

3. Results and discussion

Fig. 1 shows the plots of oxidation potentials for daidzein, genistein, enterolactone, BPA, HOPy and PCP. It was observed that four analytes (daidzein, genistein, enterolactone, and BPA) had a strong response at 560 mV. Fig. 2 shows an LC trace of a standard and a urine extract identifying these four compounds. The responses for HOPy and PCP were lower than required to detect typical concentrations of metabolites found in humans (<1 μg/L [7]). The potential plots of TCPy and nonylphenol were similar to PCP, and the response of TCPy was about 10 times lower than PCP and nonylphenol. These phenols were not investigated further.

The limits of detection and performance of the method for three phytoestrogens and BPA demonstrate that this method

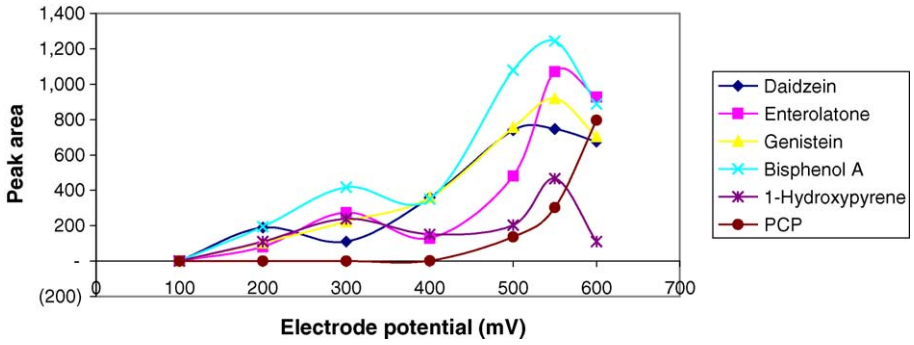

Fig. 1. Peak area ($\times 10^{-3}$) of six analytes at selected oxidation potentials (concentration 40 μg/L, E1 = 0 mV). The potential plot of TCPy was similar to PCP, but response was about 10 times lower. The response at 560 mV for nonylphenol was similar to PCP.

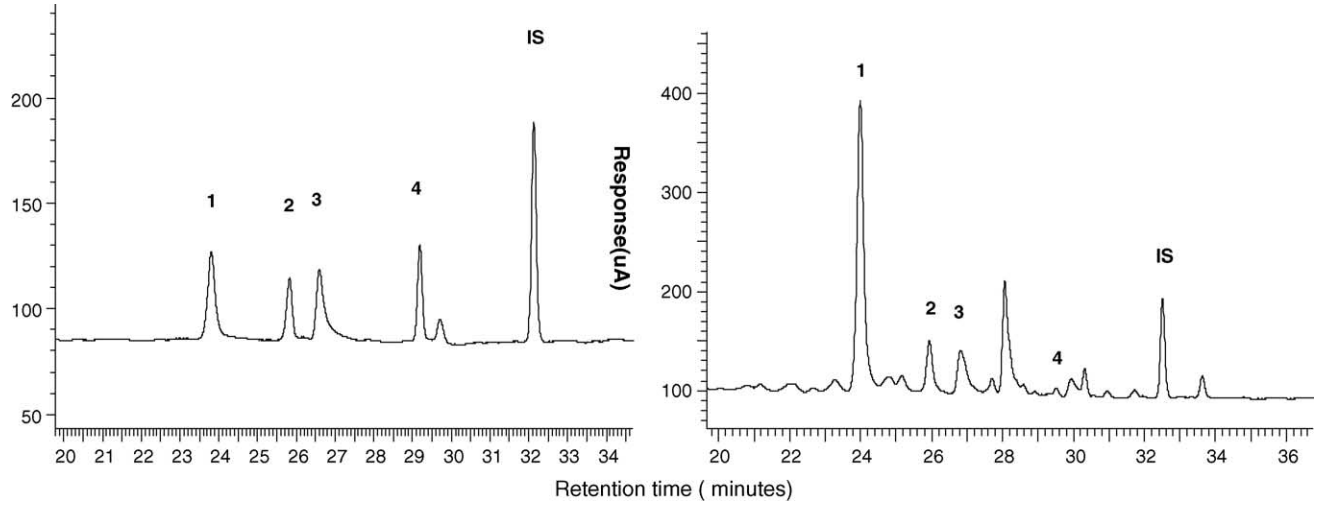

Fig. 2. LC trace of 1, daidzein; 2, enterolactone; 3, genistein; and 4, *bis*phenolA in a standard (left; approximately 80 μg/L) and in an adult human urine sample (right). The internal standard was 4,4-(2-methylpropylidene) bisphenol (284 μg/L).

is capable of assessing current exposures to these chemicals (Tables 1 and 2). LC-ECD analyses in urine or blood have been previously reported for both phytoestrogens [5,9,10] and BPA [11,12]. The detection limits (1 μg/L; Table 2) are in the range of those reported using gas chromatography-mass spectrometric (GCMS) determinations, which are considered state-of-the-art. A limitation is that the ECD method is not as specific as GCMS. It would be possible to use additional steps with LC to confirm the identity and concentration of analytes. However, UV confirmation could not be used, because the detection limits are too high for these compounds. For example, we compared the results of ECD versus UV for 15 samples (for the adults shown in Table 2). The median levels were similar for daidzein (20 versus 28 μg/L by ECD, $n = 10$) and not for genistein (20 versus 7 μg/L by ECD, $n = 15$), but the correlation between the two methods was poor (r (Spearman) = 0.55 for daidzein, 0.50 for genistein). We also examined the utility of using reduction potentials for secondary confirmation of the ECD analyses. A strong signal in the 550 mV reducing region is found for BPA with the

Table 1
Detection limits and recoveries of selected urinary biomarkers

	LD[a] (μg/L)	S.D. added (μg/L)	Recovery % in water[b]	Urine pool A mean (μg/L)[c]	Urine pool B mean (μg/L)[c]	S.D. added to urine pool A (μg/L)	Recovery % from spiked pool A[b]
Daidzein	0.4	12.8	102 ± 7	23.5 ± 5.4	474 ± 15	99.8	107 ± 5
Enterolactone	0.9	13.2	103 ± 11	176 ± 36	800 ± 197	310	95 ± 4
Genistein	0.2	12.9	99.5 ± 7	1.8 ± 0.87	62.5 ± 13.8	30.2	92 ±3
*bis*phenolA	0.5	14.9	103 ± 14	0.56 ± 0.46	0.95 ± 0.82	17.5	115 ± 7
N	9		9	10	8		9

[a] Based on three times the standard deviation of water blanks included in each batch.
[b] Based on standards diluted into water or into pool A included in each batch. Pool A recovery is corrected for the pool mean values.
[c] Two urine pools (not spiked) from various donors used for QC only.

Table 2
Summary of ECD determinations (μg/L) of four analytes in two groups and comparison with reported values by CDC or others

		This study		Other reports
		Adults	Girls	
Enterolactone	*N*	24	9	2548[a]
	%>LD	100	100	100
	Median (range)	131 (4.0–2680)	107 (27.1–182)	315 (18.8–3070)
Daidzein	*N*	24	9	2554[a]
	%>LD	83	100	100
	Median (range)	22.5 (LD –2110)	37.8 (1.8–456)	69.7 (9.3–1580)
Genistein	*N*	22	9	2557[a]
	%>LD	95	89	100
	Median (range)	2.6 (LD –356)	16.1 (0.003–27.4)	27 (0.67–734)
BPA	*N*	23	9	30–73[b]
	%>LD	52	89	61–100
	Median (range)	0.47 (LD–2.24)	2.4 (0.04–16.6)	(1–10)

[a] CDC data are for all subjects, all ages; children's urinary metabolites were generally higher than adults'.
[b] Various reports, $n = 30–73$ in US, Japan, Korea for non-exposed persons using GCMS [17] or LC with ECD [12,18,19] and fluorescence detector [14,16].

ECD and a weaker, but useable spectrum for enterolactone. The responses were 1/10th–1/5th the areas obtained with oxidation (i.e. Fig. 1), and we utilized this approach by reinjecting sample extracts to confirm identity and quantity of these two compounds in nine samples. Reduction potentials of the isoflavones were lower than for BPA and enterolactone, and were not useful for analysis. Another alternative for confirmation would be to monitor oxidation at additional potentials such as 300 mV for BPA and enterolactone. In addition, BPA is detectable at concentrations below 1 μg/L using a fluorescence detector with LC [13–16]. We did not explore this option, but fluorescence detection could be used in tandem with ECD to provide analysis and/or confirmation of these residues.

As a pilot study, we analyzed samples from two small groups (Table 2). Concerning the three phytoestrogens, more than 80% of the samples had detectable values for all three analytes. The medians were 10–100 times the limits of detection and were similar to those reported by CDC using GCMS [2]. BPA concentrations in our population were below the LD (48%) in adults and had a median of 2 μg/L among girls. Similar concentrations have been reported by other investigators, i.e. 1–3 μg/L [12,17,18]for non-occupational exposures [19] and >10 μg/L for exposures during painting [17] or following consumption of tea from contaminated containers [14]. In contrast, geometric mean concentrations of 9.5 μg/L were found among 73 Korean subjects with no known exposure [16].

The exposures discussed in this paper encompass some of the biomarkers of interest in environmental epidemiology. The three phytoestrogens and BPA possess estrogenic activity [3], and therefore their joint concentrations in humans may be relevant for risk assessment [3]. Populations with sufficiently high exposures may have detectable values of phenolic metabolites, for example values above the 95th percentile [2,20] or persons with occupational contact [21,22]. In addition, manipulation of the method to use greater volumes of urine or different ECD potentials may also enable detection of urinary metabolites other than the four described here. Finally, this method may be applicable to determination of mixtures of phenolic residues in waste water or other ecologic matrices [23,24].

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