



## A sandwich enzyme-linked immunosorbent assay for adducts of polycyclic aromatic hydrocarbons with human serum albumin

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### ABSTRACT

Adducts of benzo[*a*]pyrene-diolepoxide (BPDE) with blood nucleophiles have been used as biomarkers of exposure to polycyclic aromatic hydrocarbons (PAHs). The most popular such assay is a competitive enzyme-linked immunosorbent assay (ELISA) that employs monoclonal antibody 8E11 to detect benzo[*a*]pyrene tetrols following hydrolysis of BPDE adducts from lymphocyte DNA or human serum albumin (HSA). Here we used 8E11 as the capture antibody in a sandwich ELISA to detect BPDE–HSA adducts directly in 1-mg samples of HSA or 20  $\mu$ l of serum/plasma. The assay employs an anti-HSA antibody for detection, and this is amplified by an avidin/biotinylated horseradish peroxidase complex. The sandwich ELISA has advantages of specificity and simplicity and is approximately 10 times more sensitive than the competitive ELISA. To validate the assay, HSA samples were assayed from three populations with known high PAH exposures (coke oven workers), medium PAH exposures (steel factory control workers), and low PAH exposures (volunteer subjects) ( $n = 30$ ). The respective geometric mean levels of BPDE–HSA adducts—67.8, 14.7, and 1.93 ng/mg HSA (1010, 220, and 28.9 fmol BPDE equiv/mg HSA)—were significantly different ( $P < 0.05$ ). The sandwich ELISA will be useful for screening PAH exposures in large epidemiologic studies and can be extended to other adducts for which capture antibodies are available.

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Polycyclic aromatic hydrocarbons (PAHs)<sup>1</sup> represent a class of ubiquitous pollutants arising from combustion of hydrocarbon fuels, fires, and cigarette smoke. Several PAHs are carcinogenic in humans and animals, notably benzo[*a*]pyrene (BaP) [1]. The ultimate carcinogens derived from BaP metabolism are the isomeric benzo[*a*]pyrene-diol-epoxides (BPDEs) that bind to DNA, proteins, and other macromolecules to form adducts. Adducts of the BPDEs accumulate in blood and can be used as biomarkers of exposure to BaP and other PAHs from the same source [2].

Although DNA adducts of BPDE have been widely investigated in human populations as measures of genetic damage in human

cells, adducts of BPDE with human serum albumin (HSA) have also been detected [3]. Because HSA is much more abundant than DNA in blood (30 mg HSA/ml vs. 0.003–0.008 mg DNA/ml) and HSA adducts are not repaired, HSA adducts have inherent advantages over DNA adducts as measures of exposure for epidemiologic studies [4] even though they might not be strictly proportional to genetic damage in target organs. Furthermore, stable HSA adducts have a mean residence time of 28 days in humans that is sufficiently long to damp the day-to-day variability in exposure often observed in human studies [5]. Yet despite the relative advantages of BPDE–HSA adducts as biomarkers of exposure to PAHs, these adducts have rarely been used in epidemiologic studies.

Enzyme-linked immunosorbent assays (ELISAs) provide an efficient means for screening BPDE–HSA adducts in human studies. One such assay, developed by Santella and coworkers, employs monoclonal antibody 8E11, which was raised against benzo[*a*]pyrene-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide (BPDE-I)-modified guanosine conjugated with bovine serum albumin but cross-reacts with many other large PAHs [3]. Because this assay involves hydrolysis of BPDE adducts to the corresponding BaP tetrols, it can be applied to essentially any hydrolyzable BPDE adduct bound to proteins or DNA. As applied to HSA, the assay requires HSA to be isolated from

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<sup>1</sup> Abbreviations used: PAH, polycyclic aromatic hydrocarbon; BaP, benzo[*a*]pyrene; BPDE, benzo[*a*]pyrene-diol-epoxide; HSA, human serum albumin; BPDE–HSA, HSA adduct of BPDE; ELISA, enzyme-linked immunosorbent assay; BPDE-I, benzo[*a*]pyrene-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide; THF, tetrahydrofuran; TEA, triethylamine; TBS, Tris-buffered saline; NFD, nonfat dry milk; ABC, avidin–biotin complex; TMB, tetramethylbenzidine; HRP, horseradish peroxidase; IgG, immunoglobulin G; LC, liquid chromatograph; MS, mass spectrometer; ESI, electrospray ionization; FWHM, full width at half maximum; TBS-T, 0.05% Tween 20 in TBS; ANOVA, analysis of variance; CV, coefficient of variation; AP, alkaline phosphatase; OD, optical density; GM, geometric mean; SD, standard deviation.

blood, purified, hydrolyzed by treatment with enzymes or acid to generate BaP tetrols, purification of the tetrols, and detection of the tetrols by competitive ELISA. Although each of these steps can result in losses of analyte and can introduce imprecision, this competitive ELISA has remained essentially unchanged for 20 years [3,6,7]. Early attempts to use the 8E11-based ELISA to measure intact BPDE–HSA adducts (i.e., without hydrolysis to BaP tetrols) resulted in a 5- to 20-fold decrease in sensitivity [6,8].

Given our interest in developing high-throughput methods for screening exposures to PAHs in epidemiologic studies, we sought a simpler and more sensitive ELISA for detecting BPDE–HSA adducts in serum, plasma, or isolated HSA. Toward this end, we developed a sandwich ELISA that uses 8E11 as the capture antibody and an anti-HSA antibody for detection and requires only 1 mg of isolated HSA, equivalent to approximately 20  $\mu$ l of serum/plasma. Although simpler to use, this sandwich assay is roughly 10 times more sensitive than the original competitive ELISA. The sandwich ELISA was validated with archived human samples of HSA from workers exposed to PAHs and from control subjects.

## Materials and methods

### Chemicals and reagents

BPDE-I (MRI no. 477) was obtained from the Midwest Research Institute at the NCI Chemical Carcinogen Repository (Kansas City, MO, USA). Anhydrous tetrahydrofuran (THF,  $\geq 99.9\%$ ), carbonate–bicarbonate buffer, Tween 20, HSA (fraction V, 96–99%), and triethylamine (TEA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Tris-buffered saline (TBS, 10 $\times$ ), Tris base, acetonitrile (Fisher Optima grade, 99.9%), and formic acid (Pierce, 1-ml ampoules,  $\geq 99\%$ ) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Nonfat dry milk (NFD) was obtained from Genesee Scientific (San Diego, CA, USA). SuperBlock, ImmunoPure ABC (avidin–biotin complex) staining kits, and one-step ultra tetramethylbenzidine (TMB, the colorimetric substrate for horseradish peroxidase [HRP]) were obtained from Thermo Scientific (Rockford, IL, USA). Anti-mouse immunoglobulin G (IgG)–Fc (rabbit IgG) was obtained from Bethyl Laboratories (Montgomery, TX, USA). Anti-HSA (rabbit IgG, biotin conjugated) and anti-BPDE monoclonal antibody clone 8E11 (mouse IgG) were obtained from Rockland Immunochemicals (Gilbertsville, PA, USA) and Trevigen (Gaithersburg, MD, USA), respectively. All antibodies were polyclonal except 8E11.

### Synthesis of BPDE–HSA standards

A BPDE stock solution was prepared by dissolving BPDE-I in THF with 5% TEA under nitrogen at a concentration of 3000  $\mu$ g/ml. Commercial HSA (1 mg/ml in 0.01 M Tris buffer, pH 7.5) was mixed with BPDE stock in a molar ratio of 1:5 and gently shaken overnight in the dark at room temperature. The reaction mixture was applied to a 30,000-MW Microcon filter (Millipore, Billerica, MA, USA), washed three times with 400  $\mu$ l of 0.01 M Tris buffer, and diluted to a concentration of 1 mg/ml with 0.01 M Tris buffer (pH 7.5). For quantitation purposes, BPDE equivalents were estimated assuming a molecular weight of 66,863 fg of BPDE–HSA/fmol, as estimated by mass spectrometry of the BPDE–HSA standard (described below), and 1 mol of BPDE/mol of BPDE–HSA.

### Mass spectrometric characterization of BPDE–HSA adducts

Samples of commercial HSA before and after reaction with BPDE were characterized with an Agilent 1200 series liquid chromatograph (LC, Santa Clara, CA, USA) that was connected in-line with an LTQ Orbitrap XL hybrid mass spectrometer (MS) equipped with an Ion Max electrospray ionization (ESI) source (Thermo Fisher Sci-

entific, Waltham, MA, USA). The LC employed C8 guard (Poroshell 300SB–C8, 5  $\mu$ m, 12.5  $\times$  2.1 mm, Agilent) and analytical (75  $\times$  0.5-mm) columns and a 100- $\mu$ l sample loop. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile (v/v). Following sample injection, analyte trapping was performed for 5 min with 99.5% A at a flow rate of 90  $\mu$ l/min. The elution program consisted of a linear gradient from 25 to 95% B over 34 min, isocratic conditions at 95% B for 5 min, a linear gradient to 0.5% B over 1 min, and then isocratic conditions at 0.5% B for 14 min at a flow rate of 90  $\mu$ l/min. The column and sample compartments were maintained at 35 and 10  $^{\circ}$ C, respectively. The MS ESI source parameters were as follows: ion transfer capillary temperature, 275  $^{\circ}$ C; normalized sheath gas (nitrogen) flow rate, 25%; ESI voltage, 2.0 kV; ion transfer capillary voltage, 33 V; and tube lens voltage, 125 V. Positive ion mass spectra were recorded over the range  $m/z$  500 to 2000 using the Orbitrap mass analyzer, in profile format, with full MS automatic gain control target settings of  $3 \times 10^4$  and  $5 \times 10^5$  charges for the linear ion trap and Orbitrap, respectively, and an Orbitrap resolution setting of  $6 \times 10^4$  (at  $m/z$  400, full width at half maximum [FWHM]). ESI mass spectra were processed using Xcalibur software (version 4.1, Thermo Fisher Scientific), and charge state distributions were deconvoluted using ProMass software (version 2.5 SR-1, Novatia, Monmouth Junction, NJ, USA) with the default “large protein” parameters and a background subtraction factor of 1.5.

### HSA samples

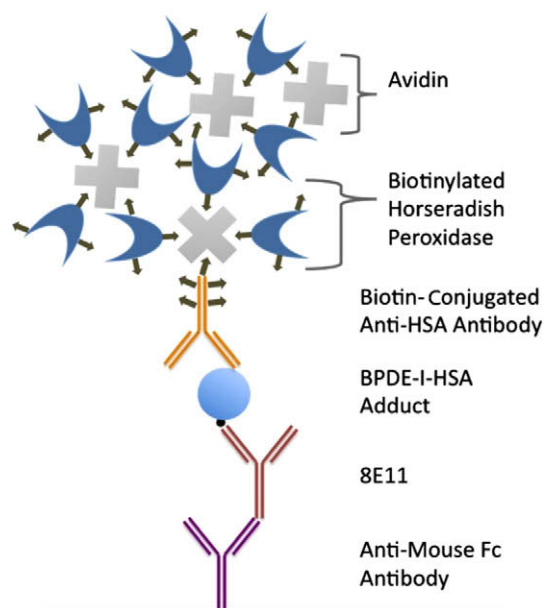
For validation of the ELISA, 30 archived specimens of HSA from both sexes were obtained from studies of PAH-exposed workers at a steel factory in Northern China [9] and volunteer control subjects from the state of North Carolina, USA [10]. All blood samples had been obtained with informed consent after approval of protocols by institutional review boards at the institutions where the initial investigations were conducted. The PAH-exposed subjects consisted of 10 top-side coke oven workers and 10 factory control workers (both smokers and nonsmokers) from the same steelmaking complex in Northern China; these workers had previously been shown to have high and intermediate levels of urinary PAH metabolites, respectively [11]. The volunteer control subjects were nonsmokers. All HSA specimens had previously been isolated from plasma, dialyzed, lyophilized to constant weight, dissolved in distilled water (50 mg/ml), and stored at  $-80^{\circ}$ C prior to analysis.

### Standard ELISA procedures

Unless otherwise specified, standard washing steps were applied throughout the assay, and all reagents, antibodies and HSA samples or standards were loaded at 100  $\mu$ l/well in 96-well plates (MaxiSorp, C type, Nunc, Rochester, NY, USA). Prior to loading with analytes, plates were rinsed and briefly vortexed three times with 200  $\mu$ l of TBS-T (0.05% Tween 20 in TBS) on a microplate mixer (MicroMix 5, DPC, Flanders, NJ, USA). After loading, plates were vortexed briefly, incubated for 1.5 h (45 min for reduced-volume ELISA) at 37  $^{\circ}$ C, and rinsed as described above. Wells were blocked at 37  $^{\circ}$ C with 250  $\mu$ l/well of either 5% NFD, SuperBlock, or 15% NFD as indicated. A one-step TMB solution was added, and plates were incubated for 45 min before stopping the reaction by the addition of 100  $\mu$ l/well of 2 M sulfuric acid. Colorimetric measurements of TMB were made at 450 nm using a microplate spectrophotometer (ELx800, Bio-Tek, Winooski, VT, USA).

### Sandwich ELISA design

The sandwich ELISA design is illustrated in Fig. 1. Anti-mouse IgG–Fc (rabbit IgG) at 5  $\mu$ g/ml in 0.1 M carbonate–bicarbonate



**Fig. 1.** Illustration of the sandwich ELISA design. The design incorporates an anti-mouse IgG-Fc to increase the effective concentration of 8E11. Signals are amplified with an ABC–HRP detection system.

buffer was coated into the 96-well plate at 4 °C overnight. After blocking with SuperBlock, monoclonal antibody 8E11 at 0.5 µg/ml was added and the plate was incubated. After loading standards of BPDE–HSA adducts into the wells and incubating, biotin-conjugated anti-HSA (rabbit IgG) at 1 µg/ml in blocking buffer was loaded and the plate was incubated again. ABC reagent, prepared in TBS-T, was added to the wells, and the plate was incubated for 30 min at room temperature and then rinsed five times.

#### Reduced-volume ELISA

To reduce background signals from unadducted HSA, the sandwich ELISA was modified slightly by reducing the volume of reagents to 20 µl/well. After coating the 96-well plate (50 µl/well) with anti-mouse IgG-Fc (rabbit IgG) diluted to 1 µg/ml with 0.1 M carbonate–bicarbonate buffer, the plate was incubated at 4 °C overnight. The plate was blocked with 15% NFDm dissolved in TBS-T, and monoclonal antibody 8E11 at 3 µg/ml in blocking buffer was added. After incubating the plate, the BPDE–HSA adduct sample or unmodified HSA sample was loaded in a 20-µl volume and the plate was incubated for 1.5 h. The assay then proceeded as described above except that biotin-conjugated antibody was prepared with SuperBlock and 8.5 M acetic acid and 0.5 M sulfuric acid (10 µl) was used to stop the final reaction.

This reduced-volume ELISA was used to measure BPDE–HSA adducts in samples of HSA from PAH-exposed workers and volunteer control subjects (in duplicate) after blinding of the analyst as to exposure status and randomization of samples. Wells containing sample HSA without monoclonal antibody 8E11 were used as individual controls for all specimens of HSA.

#### Statistical analyses

Dose–response curves were fitted by the variable slope sigmoid function,  $y = \min + \frac{\max - \min}{1 + 10^{(\log IC_{50} - x) \cdot \text{Hillslope}}}$ , where  $y$  is the absorbance,  $\min$  is the minimum response plateau,  $\max$  is the maximum response plateau,  $\log IC_{50}$  is the log (base 10) of  $IC_{50}$ ,  $x$  is the log (base 10) concentration of BPDE–HSA, and  $\text{Hillslope}$  is the slope of the curve (QtiPlot, ProIndep Serv, Craiova, Romania). Descriptive statistics

and pairwise  $t$  tests with Bonferroni correction (by one-way analysis of variance [ANOVA]) were performed with STATA 10 (Stata-Corp, College Station, TX, USA) using log-transformed (base  $e$ ) data to satisfy normality assumptions. Normality and homogeneity of variance were confirmed by Shapiro–Wilks and Bartlett’s tests, also using STATA 10. Values below the detection limit were imputed a value of half the detection limit for statistical analyses.

Limits of detection and quantitation were defined, respectively, as the mean of 12 blank values obtained with BPDE–HSA standards and spiked samples of either 1 mg of control HSA or 20 µl of control serum plus either 3 or 10 times the respective standard deviation [12]. The following respective detection and quantitation limits for the reduced-volume ELISA were estimated: standards, 0.124 and 0.619 ng of BPDE–HSA (1.80 and 9.26 fmol BPDE equiv); 1 mg of HSA, 0.704 and 2.310 ng of BPDE–HSA (10.5 and 34.5 fmol BPDE equiv); and 20 µl of serum, 0.637 and 2.540 ng of BPDE–HSA (9.52 and 38.00 fmol BPDE equiv). To estimate the precision of the assay, duplicate reference standards at 5 and 80 ng of BPDE–HSA/well (75 and 1200 fmol BPDE equiv/well) were prepared in each plate on 5 different days. The coefficients of variation (CVs) estimated from one-way ANOVA of the five data pairs (in natural scale) were as follows: intraassay, 8.71% (80 ng/well) and 32.2% (5 ng/well); and interassay, 10.7% (80 ng/well) and 15.0% (5 ng/well).

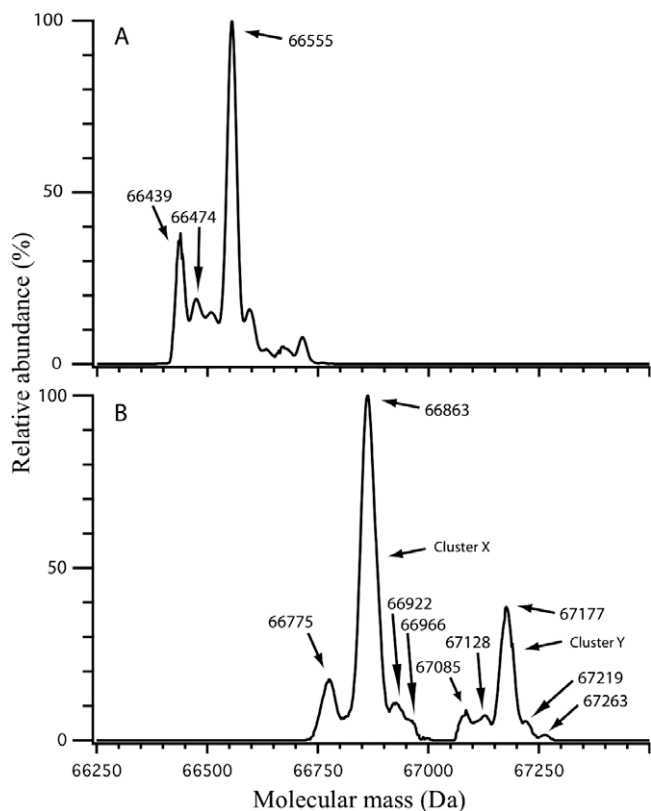
## Results and discussion

### Characterization of BPDE–HSA standard

We quantified the modification level of the BPDE–HSA standard by ESI mass spectrometry of the intact proteins, as illustrated in Fig. 2. There was little overlap between deconvoluted mass spectra of commercial HSA (Fig. 2A) and the BPDE–HSA standard obtained by reaction of commercial HSA with BPDE-I (Fig. 2B), suggesting that the standard contained BPDE–HSA adducts in high yield. The most prominent mass in the spectrum of commercial HSA (Fig. 2A) was 66,555 Da, representing the cysteinylated adduct at HSA–Cys34, whereas mercaptalbumin was represented by the smaller peak at 66,439 Da [13]. In contrast, the mass spectrum of the BPDE–HSA standard was dominated by peaks at 66,863 and 67,177 Da, which are labeled as clusters X and Y, respectively, in Fig. 2B. The mass differences between clusters X and Y and the prominent peak in commercial HSA were 308 Da (66,863 – 66,555 Da) and 622 Da (67,177 – control HSA at 66,555 Da), respectively. Because the theoretical mass change for the addition of 1 mol of BPDE to 1 mol of HSA is 302.3 Da, we conclude that cluster X most likely represents 1 BPDE adduct/HSA molecule and cluster Y most likely represents 2 BPDE adducts/HSA molecule. Based on the relative abundances of the corresponding ions in the raw spectra (not shown), roughly 80% of the BPDE–HSA standard contained a single BPDE modification and 20% contained two BPDE modifications. Thus, the average level of BPDE modification was approximately 1.2 mol of BPDE/mol of HSA. Because stable covalent BPDE binding has been observed at His146 of HSA and relatively unstable ester adducts have been observed at Asp187 and Glu188 of HSA [14,15], it is reasonable to expect that more than one BPDE molecule could be bound to a single HSA molecule. Also, because repeated analysis of the BPDE–HSA standard showed that adducts were stable under prolonged periods of laboratory storage (results not shown), it appears that most of the adducts in our standard represent the stable modification at His146 of HSA.

### Response of the sandwich ELISA

The sandwich ELISA (Fig. 1) incorporated several elements to enhance sensitivity of the original competitive ELISA format. These in-

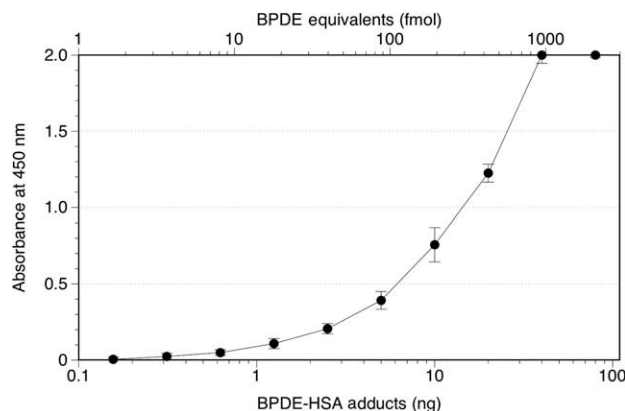


**Fig. 2.** Deconvoluted mass spectra of commercial HSA (A) and the BPDE-HSA standard (B). Masses in panel A at 66,439 and 66,555 Da are mercaptalbumin and HSA, respectively, the latter of which has been cysteinylated at HSA-Cys34. The mass difference between cluster X in panel B at 66,683 Da and cysteinylated HSA in panel A is 308 Da, and the mass difference between cluster Y in panel B at 67,177 Da and cysteinylated HSA is 622 Da. Because the theoretical mass change after the addition of 1 mol of BPDE to 1 mol of HSA is 302.3 Da, clusters X and Y probably represent cysteinylated HSA that has been modified with one and two BPDE molecules, respectively.

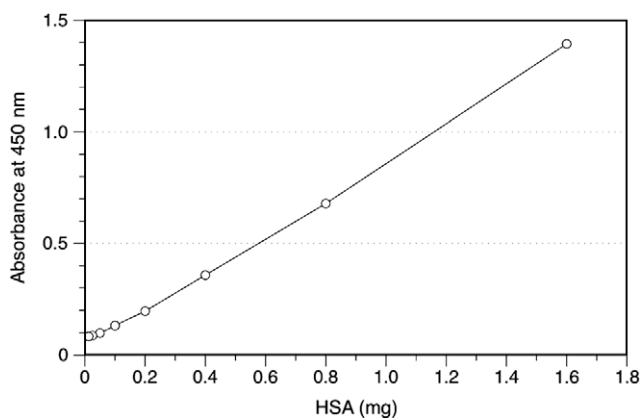
cluded coating the wells with a tether antibody, anti-mouse IgG-Fc (rabbit IgG), and substituting an ABC-HRP detection system for the alkaline phosphatase (AP)-*p*-nitrophenylphosphate system. By coating the wells with a tether antibody, we sought to minimize denaturation and loss of specificity [16] and also to free the 8E11 capture antibody from any constraints that might be imposed by its direct attachment to the wells. Introduction of the ABC-HRP detection system to replace AP-*p*-nitrophenylphosphate should theoretically amplify the detection of BPDE-HSA adducts by a factor of 4 because one avidin molecule is capable of binding four biotin molecules. The response curve is shown in Fig. 3. With 10 ng of BPDE-HSA/well (150 fmol BPDE equiv/well), the sandwich ELISA produced an absorbance of 0.76 optical density (OD) units and the detection limit for standard solutions was estimated to be 0.1 ng of BPDE-HSA/well (1.5 fmol BPDE-HSA equiv/well). Attempts to increase sensitivity by substituting a fluorescent AP substrate (Atto-Phos, Promega, Madison, WI, USA) [17] for *p*-nitrophenylphosphate were unsuccessful (data not shown).

#### Reducing background effects of HSA

Because adduct levels in HSA are expected to be approximately 1 molecule of BPDE-HSA/ $10^6$  HSA molecules, unadducted HSA can produce substantial background signals due to nonspecific binding of HSA to the antibodies and internal surfaces of the wells. To illustrate this effect, Fig. 4 shows the increase in background signals with increasing quantities of control HSA added to each well. We

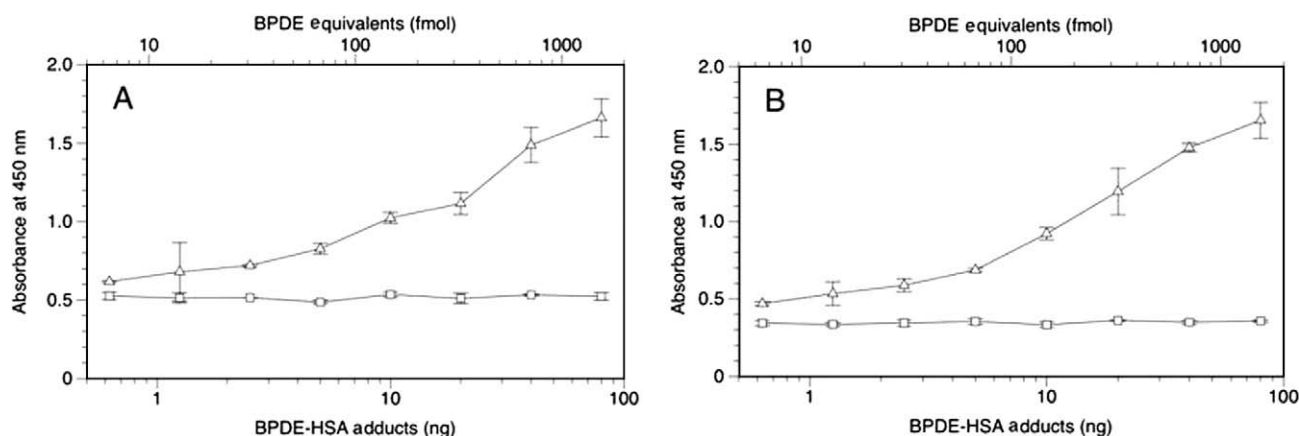


**Fig. 3.** Standard curve obtained from the sandwich ELISA using 1 mg of HSA/well spiked with increasing amounts of the BPDE-HSA standard. Error bars represent standard deviations of duplicate samples.



**Fig. 4.** Relative background signals from nonspecific binding of HSA in the sandwich ELISA. Reactions were stopped at 8 min (rather than 45 min) to avoid signal saturation.

sought to reduce this background signal by reducing the total reagent volume from 100 to 20  $\mu$ l, thereby reducing the effective surface area of each well while also increasing the concentration of NFD from 5 to 15% in the blocking buffer (to increase competition of NFD with HSA for nonspecific binding). Fig. 5 shows response curves for this modified protocol when applied to 1-mg samples of HSA spiked with BPDE-HSA (Fig. 5A) or 20  $\mu$ l of human serum spiked with BPDE-HSA (Fig. 5B). (Note that 20  $\mu$ l of human serum contains approximately 0.84 mg of HSA.) In both Fig. 5A and B, signals from control samples of 1 mg of HSA or 20  $\mu$ l of serum (without the addition of 8E11) did not increase with increasing amounts of BPDE-HSA adducts. Also, a stable background of approximately 0.5 OD units was attained after only 45 min of incubation, which was sufficient for the assay to achieve full sensitivity. These results indicate that modifications to the sandwich ELISA effectively controlled the background signal and increased the signal-to-noise ratio in the presence of high concentrations of unadducted HSA. The detection limit after the modification is approximately 0.7 ng of BPDE-HSA/mg of HSA (10 fmol BPDE equiv/mg HSA). This can be compared with an estimated detection limit of 10 to 20 ng of BPDE-HSA/mg of HSA (150–300 fmol BPDE equiv/mg HSA) for the conventional competitive ELISA [7,18]. (Because the competitive ELISA is based on measurement of BaP tetrols that are extracted with variable efficiency after being liberated from adducts, actual detection limits are difficult to estimate for this assay.)



**Fig. 5.** Response curves obtained from the low-volume ELISA design using either 1 mg of HSA/well (A) or 20  $\mu$ l of human serum/well (B) spiked with increasing amounts of the BPDE–HSA standard.  $\Delta$ , BPDE–HSA adducts;  $\square$ , controls (without 8E11). HSA solutions were prepared at 50 mg/ml. Both HSA and serum contained 15% NFDM and 0.1% Tween 20. Error bars represent standard deviations of duplicates.

#### Measuring BPDE–HSA adducts in human samples

To validate the sandwich assay, archived HSA samples from subjects exposed to high, medium, and low (control) levels of PAH were randomized and assayed with the analyst blinded to exposure status. Descriptive statistics are shown in Table 1 for the assays of PAH-exposed and control subjects. Adducts were detected in all 20 PAH-exposed workers (high- and medium-exposure groups) and in 7 of 9 volunteer control subjects. Adduct levels spanned a 200-fold range across specimens of HSA from workers with high and medium exposure to PAHs (1.4–257 ng BPDE–HSA/mg HSA equiv to 21–3850 fmol BPDE equiv/mg). Pairwise *t* tests detected significant differences across the three groups ( $P < 0.05$  for all pairwise tests). These results demonstrate that the optimized sandwich ELISA can be used as a screening tool to quantify PAH exposures in PAH-exposed and control populations.

It is informative to compare levels of BPDE–HSA adducts from our investigation with those reported in previous studies that used the competitive ELISA to measure BPDE–HSA adducts in PAH-exposed workers and controls. We found four such studies: Kure and coworkers [19], who reported adduct levels in coke oven workers and rural controls; Lee and coworkers [3], who reported adduct levels in foundry workers, roofers, and controls; and Sheron and coworkers [20] and Omland and coworkers [21], who reported adduct levels in foundry workers and controls. Results from these studies and the current investigation are summarized in Table 2. For comparison, geometric mean (GM) or median adduct levels are shown as fmol of BPDE equiv/mg of HSA. For the Kure and coworkers and Lee and coworkers studies, GM concentrations were estimated from the reported mean and standard deviation (SD) values according to the following relationship:  $GM = (\text{mean})^2 / \sqrt{\text{mean}^2 + \text{SD}^2}$ . Absolute adduct concentrations varied widely across the published studies, even in nonsmoking control subjects, where levels ranged from 28.9 fmol of BPDE equiv/mg of HSA in the current study to 3280 fmol of BPDE

equiv/mg of HSA in the Lee and coworkers study. To reduce effects of interlaboratory differences, Table 2 also shows fold ratios of BPDE–HSA levels measured in each group of exposed workers compared with those measured concurrently in control subjects. Using the sandwich assay, we found fold ratios of 35 for the coke oven workers and 7.6 for the steel factory control workers compared with controls. Although these fold ratios are considerably larger than those from the other studies, which used the competitive ELISA (fold ratio range = 0.83–5.60), the results should be regarded as preliminary given the small numbers of subjects involved and the difficulties in choosing control subjects for PAHs, which are ubiquitous contaminants of air, water, food, and tobacco smoke. Nonetheless, the combination of lower control levels of BPDE–HSA adducts and higher fold ratios observed with the sandwich ELISA suggests that this assay will be useful for characterizing PAH exposures in epidemiologic investigations.

#### Specificity of ELISA for BPDE–HSA adducts

Although 8E11 is a monoclonal antibody raised against BPDE-modified antigens, it is known to cross-react with a range of PAHs and PAH adducts, particularly those with four or more rings [3]. Even though such cross-reactivity is not necessarily a disadvantage, in the sense that exposures to PAHs rather than to BPDE are generally of interest, it follows that levels of BPDE–HSA adducts per se can be overestimated by any 8E11-based ELISA. However, the extent of overestimation should be greater for the competitive ELISA than for the sandwich ELISA because the sandwich design is specific for HSA adducts and cannot detect free PAH tetrols that can arise from both BPDE–HSA adducts and other sources. Some support for the increased specificity of the sandwich design is given in Table 2, where the level of BPDE equivalents measured in control subjects with the sandwich ELISA (GM = 28.9 fmol BPDE equiv/mg HSA) was roughly 100-fold less than the median value

**Table 1**  
Concentrations of BPDE–HSA adducts in groups of PAH-exposed and unexposed persons from this study.

Exposure group	PAH exposure	n	ng of BPDE–HSA/mg of HSA and fmol of BPDE equiv/mg of HSA		
			Geometric mean	Geometric SD	Range
Coke oven workers	High	10	67.80* (1010)	1.99	22.4–257 (336–3800)
Steel factory control workers	Medium	10	14.70* (220)	2.63	1.4–53.5 (21.0–801)
Unexposed controls <sup>a</sup>	Low	10	1.93* (28.9)	2.68	0.5–15.3 (7.5–229)

Note. The fmol of BPDE equiv/mg of HSA values are in parentheses.

\*  $P < 0.05$  versus both other groups. Comparisons were made by ANOVA of logged data using pairwise *t* tests with Bonferroni correction.

<sup>a</sup> Adducts not detected (<1 ng/mg HSA) in 2 control subjects.

**Table 2**  
Equivalent concentrations of BPDE–HSA adduct from this study and from previous studies of PAH-exposed workers and control subjects that employed the competitive ELISA format.

Study	ELISA	Exposure group	Location	Number of subjects	BPDE equivalents (fmol/mg HSA)	Fold ratio (exposed/controls)
Current	Sandwich	Coke oven workers (smoking and nonsmoking)	China	10	1010 (GM)	35
	Sandwich	Steel factory control workers (smoking and nonsmoking)	China	10	220 (GM)	7.6
	Sandwich	Unexposed controls (nonsmoking)	USA	10	28.9 (GM)	
Kure et al. [19]	Competitive	Coke oven workers (smoking and nonsmoking)	Norway	38	3920 (GM)	1.5
	Competitive	Controls (smoking and nonsmoking)	Norway	45	2620 (GM)	
Lee et al. [3]	Competitive	Foundry workers (nonsmoking)	Finland	13	5020 (GM)	1.4
	Competitive	Controls (nonsmoking)	Finland	10	3600 (GM)	
	Competitive	Roofers (nonsmoking)	USA	12	4020 (GM)	1.5
	Competitive	Controls (nonsmoking)	USA	12	3280 (GM)	
Omland et al. [21]	Competitive	Foundry workers (nonsmoking)	Denmark	25	580 (median)	0.83
	Competitive	Controls (nonsmoking)	Denmark	26	700 (median)	
Sherson et al. [20]	Competitive	Foundry workers (nonsmoking)	Denmark	19	200 (median)	5.6
	Competitive	Controls (nonsmoking)	Denmark	19	35.9 (median)	

Note. GM, geometric mean.

for controls in the four studies that used the competitive ELISA (2620 fmol BPDE equiv/mg HSA).

The specificity of the sandwich ELISA also encourages the use of human serum or plasma rather than purified HSA for applications in epidemiologic studies. Although direct assays of serum or plasma simplify sample processing, they also open the possibility that binding between 8E11 and PAH adducts may be reduced by matrix effects. Although we saw no signs of such effects in preliminary experiments (Fig. 5), more extensive testing of serum and plasma versus purified HSA is warranted in the various populations and specimens of interest.

#### Extending the sandwich ELISA to other HSA adducts

Competitive ELISAs have been used to quantify a number of protein adducts associated with different chemical exposures, including formaldehyde [22], aflatoxin [23], and other mycotoxins [24]. It is reasonable to expect that our sandwich ELISA design could easily be extended to other HSA adducts by using existing antibodies to capture adducts of interest. Also, a sandwich ELISA that incorporates an adduct-specific capture antibody can be applied to advanced platforms for automation and simultaneous measurement of multiple adducts. For example, a lab-on-a-chip microfluidic device with automated loading and mixing of reagents would vastly increase the throughput of ELISAs for HSA adducts. Furthermore, the low-volume requirement of the sandwich ELISA (20  $\mu$ l serum/plasma) would minimize the use of valuable archived specimens.

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