

Hemoglobin and albumin adducts of benzene oxide among workers exposed to high levels of benzene

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Benzene oxide (BO) reacts with cysteinyl residues in hemoglobin (Hb) and albumin (Alb) to form protein adducts (BO-Hb and BO-Alb), which are presumed to be specific biomarkers of exposure to benzene. We analyzed BO-Hb in 43 exposed workers and 42 unexposed controls, and BO-Alb in a subsample consisting of 19 workers and 19 controls from Shanghai, China, as part of a larger cross-sectional study of benzene biomarkers. The adducts were analyzed by gas chromatography-mass spectrometry following reaction of the protein with trifluoroacetic anhydride and methanesulfonic acid. When subjects were divided into controls ($n = 42$) and workers exposed to ≤ 31 ($n = 21$) and >31 p.p.m. ($n = 22$) benzene, median BO-Hb levels were 32.0, 46.7 and 129 pmol/g globin, respectively (correlation with exposure: Spearman $r = 0.67$, $P < 0.0001$). To our knowledge, these results represent the first observation in humans that BO-Hb levels are significantly correlated with benzene exposure. Median BO-Alb levels in these 3 groups were 103 ($n = 19$), 351 ($n = 7$) and 2010 ($n = 12$) pmol/g Alb, respectively, also reflecting a significant correlation with exposure (Spearman $r = 0.90$, $P < 0.0001$). The blood dose of BO predicted from both Hb and Alb adducts was very similar. These results clearly affirm the use of both Hb and Alb adducts of BO as biomarkers of exposure to high levels of benzene. As part of our investigation of the background levels of BO-Hb and BO-Alb found in unexposed persons, we analyzed recombinant human Hb and Alb for BO adducts. Significant levels of both BO-Hb (19.7 pmol/g) and BO-Alb (41.9 pmol/g) were detected, suggesting that portions of the observed background adducts reflect an artifact of the assay, while other portions are indicative of either unknown exposures or endogenous production of adducts.

Abbreviations: Alb, albumin; ANOVA, analysis of variance; BO, benzene oxide; BO-Hb and BO-Alb, adducts resulting from reaction of BO with cysteinyl residues in Hb and Alb, respectively; GC-MS, gas chromatography-mass spectrometry; Hb, hemoglobin; PTTA, phenyltrifluoroacetate; rHA, recombinant human Alb; rHb0.1, recombinant human Hb; SPC, S-phenylcysteine; TWA, time-weighted average.

Introduction

Although exposure to benzene is associated with hematotoxicity including leukemia in humans and cancers in laboratory animals, the mechanism by which benzene exerts these effects remains unknown (1). To help characterize the relationship between exposure and effects in benzene-exposed workers, a cross-sectional study of 44 exposed workers and 44 unexposed controls was undertaken as described by Rothman *et al.* (2,3). This study is being used to investigate a variety of biomarkers of exposure [protein adducts (reported herein) and urinary metabolites (2)], susceptibility [cytochrome P4502E1 and NQO1 (NAD(P)H:quinone oxidoreductase) polymorphisms (4)], and early biological effects [hematotoxicity (2), somatic cell mutations (3) and chromosomal damage (5)].

Biomarkers of exposure are expected to improve the ability to elucidate the exposure-response relationship because they take into consideration factors such as individual metabolism and personal habits [including the use of protective equipment and non-work-related exposures (6)]. While both urinary metabolites and protein adducts of carcinogens (or their reactive metabolites) are thought to represent specific biomarkers of exposure, urinary metabolites indicate only recent exposures (h), whereas protein adducts represent longer-term exposures (months). Since human albumin (Alb) has a half-life of 20–25 days and human hemoglobin (Hb) has a lifespan of ~120 days (corresponding to the turnover of red blood cells), stable adducts of either protein are representative of exposures occurring over the 1–4 month period prior to blood collection (7).

Benzene is readily metabolized to the benzene oxide (BO), which can bind to the nucleophilic sites (e.g. the thiol moiety of cysteine) of proteins such as Hb and Alb, yielding BO-Hb and BO-Alb. Benzene can also give rise to a number of phenolic metabolites, including phenol, catechol, hydroquinone and 1,2,4-benzenetriol (1,8). These compounds can be further oxidized to their corresponding quinones, which are also capable of binding to nucleophilic sites on proteins. Whereas high levels of background adducts of 1,2- and 1,4-benzoquinone have been detected in ostensibly unexposed persons and animals (9–12), protein adducts of BO are expected to be more specific for benzene exposure (13,14). Bechtold *et al.* (15) showed that cysteinyl BO-Alb was correlated with benzene exposure in subjects occupationally exposed to 4.4–23 p.p.m. benzene. However, BO-Hb was not detected in the same subjects, probably due to inadequate sensitivity of the assay (limit of detection ≈ 500 pmol/g Hb) (13). We developed a more sensitive method for BO-Hb (limit of detection ≈ 20 pmol/g Hb) (14), which has recently been applied to both BO-Hb and BO-Alb in rats dosed with benzene (16).

It is interesting to note that protein adducts of BO (13–16) as well as other reactive metabolites, such as 1,2- and 1,4-benzoquinone (9–12), styrene-7,8-oxide (17,18), ethylene oxide (19), 4-aminobiphenyl (20), acrylamide (21) and buta-

diene metabolites (22), have been detected in humans and animals without obvious exposures to these reactive species or their precursors. It is important to determine whether these background adducts represent unknown or endogenous exposures, or are merely artifacts of the analytical methods. Part of the difficulty relates to the designation of negative controls (i.e. 'blank' proteins), since all humans and animals are exposed to a variety of small organic molecules in air, food and water. Recently, recombinant DNA techniques have enabled the production of both recombinant human Alb (rHA) and recombinant human Hb (rHb0.1) in bacteria and/or yeast (23–25). These recombinant proteins should represent valid negative controls and can shed light on the possible origins of background adducts.

The purpose of this research was to analyze BO-Hb in 44 currently exposed workers and 44 controls and BO-Alb in a subset of 20 workers and 20 controls, and to correlate the adduct levels with the estimated individual benzene exposures. Combined with recently determined kinetic data for the reaction of BO with cysteinyl residues in Hb and Alb (16), the adduct levels were used to predict the average concentration of BO expected in the blood of these exposed workers. An additional goal was to explore the origins of background BO adducts detected by measuring BO-Hb and BO-Alb in recombinant human Hb and Alb.

Materials and methods

Reagents and proteins

rHA was kindly provided by Delta Biotechnology (Nottingham, UK). rHb0.1 was kindly provided by Somatogen (Boulder, CO). rHb0.1 is produced in *E.coli* and differs from wild type Hb in that the two α -globin subunits are fused, and methionine rather than valine is the N-terminal residue of both the α - and β -subunits (23,25). Human Hb and Alb were obtained from Sigma (St Louis, MO). [$^2\text{H}_5$]S-phenylcysteine (SPC) was synthesized by A.Gold and R.Sangaiah (University of North Carolina at Chapel Hill). Methanesulfonic acid was purchased from Fluka (Switzerland) and trifluoroacetic anhydride was obtained from Pierce (Rockford, IL), and was distilled twice before use.

Subjects

Forty-four workers currently exposed to a wide range of benzene concentrations were recruited from three factories in Shanghai, China. In Factory 1, workers used benzene to solubilize natural rubber for subsequent production of rubber padding for printing presses; in Factory 2, benzene was used to manufacture adhesive tape; and in Factory 3, benzene-based paint was applied to wooden toys and boxes. Forty-four controls were selected from two factories (a sewing machine manufacturing plant and an administrative facility) that did not use benzene or other hematotoxic agents, and were located in the same geographic area. The controls were frequency-matched to the exposed subjects on gender and age (5-year intervals). More details about the study population have been previously reported (2).

Here, we report BO-Hb levels for 43 (of 44) exposed workers and 42 (of 44) unexposed controls and BO-Alb levels for a subset consisting of 19 (of 20) workers selected to represent a wide range of current benzene exposure and 19 (of 20) controls frequency matched to exposed workers by age and gender. At the time this study was undertaken, only 40 Alb samples were available for analysis. Subsequent work will include Alb adduct data for the entire cohort of 88 workers. The missing samples were due to insufficient availability of protein or loss during sample analysis.

Exposure assessment

Individual exposures were monitored using passive personal monitors worn by each exposed worker during the full workshift on five separate days during the 1–2 week period prior to blood collection (2). The geometric mean of the five air measurements was used to estimate the individual median daily [8 h time-weighted average (TWA) in p.p.m.] exposure to benzene (note that 1 p.p.m. = 3.2 mg benzene/m³ air). It should be noted that 16 workers in Factory 1 used half-mask charcoal respirators during the highest exposure period of their workshift, which lasted ~3 h per shift. Overall, the workers were exposed to a median 8 h TWA of 31 p.p.m. benzene (2).

Subjects in the control factory (where sewing machines were manufactured)

were monitored for benzene exposure on a single day. Subjects in the administrative facility were assumed to have no exposure to benzene (2).

Analysis of BO-Hb and BO-Alb

BO-Hb and BO-Alb were analyzed using the procedure developed by Yeowell-O'Connell *et al.* (14) for BO-Hb and extended to BO-Alb (16) with minor modifications. Alb was isolated from plasma by ammonium sulfate precipitation of the immunoglobulins followed by dialysis of the supernatant (18). Most of the globin was isolated from Hb using dialysis to remove small molecules prior to precipitation of the globin (16); however, 10 samples had been purified earlier using Sephadex chromatography (9). There were enough red blood cells to reisolate the globin for five of these 10 samples using the dialysis-based procedure.

Following isolation, 5 mg globin (apohemoglobin) or 4 mg Alb and 3–5 pmol [$^2\text{H}_5$]SPC (internal standard) were dried in a vacuum oven. Eight hundred microlitres of trifluoroacetic anhydride and 20 μl methanesulfonic acid were added and the reaction mixture was heated to 100°C for 40 min. The remaining trifluoroacetic anhydride was removed under a stream of nitrogen and 1 ml hexane was added. The hexane layer was washed with 1 ml of 0.1 M Tris buffer, pH 7.5, and 2 $\times$ 1 ml deionized water. After concentrating the hexane layer to ~200 μl , 2–3 μl of each sample were analyzed by gas chromatography-mass spectrometry (GC-MS) in the negative-ion chemical-ionization mode using selected ion monitoring for the derivatized analyte, phenyltrifluoroacetate (PTTA, m/z 206), and the derivatized internal standard ([$^2\text{H}_5$]PTTA, m/z 211; ref. 14).

In order to estimate the precision of both the overall assay and the GC-MS step for the analysis of human BO-Hb adducts, Hb samples from five subjects were carried through the entire assay (including globin isolation, derivatization and GC-MS analysis) in duplicate, and 32 final extracts were re-injected for detection by GC-MS. For those subjects with replicate analyses, the sample mean of all measurements was used for subsequent statistical comparisons.

In addition to the proteins from the Chinese workers, five 5-mg aliquots of Sigma Hb, four 5-mg aliquots of rHb0.1, four 4-mg aliquots of Sigma Alb and four 4-mg aliquots of rHA were analyzed for BO adducts as described above. Globin was isolated from Sigma Hb or rHb0.1 in the same way (with dialysis) as were the experimental samples.

Prediction of the average concentration of BO in blood

Assuming that all adduction of Alb takes place in the blood and that the BO-protein adducts are stable *in vivo*, then it is possible to predict the average blood concentration of BO ([BO]) that would be expected per unit exposure to benzene in a typical worker from this cohort (26). As described in Lindstrom *et al.* (16), BO-Alb adduct levels were used to calculate [BO] as follows:

$$[\text{BO}] = \frac{[\text{BO} - \text{Alb}]_{\text{ss}} \cdot k_{\text{Alb}}}{[\text{Alb}] \cdot k_{\text{BO} - \text{Alb}}} \quad (1)$$

where $[\text{BO} - \text{Alb}]_{\text{ss}}/[\text{Alb}]$ (in pmol/g Alb) represents the steady state BO-Alb adduct level, k_{Alb} (in h) is the first-order elimination rate constant for the turnover of Alb, and $k_{\text{BO} - \text{Alb}}$ [in l/g Alb/h] is the second-order rate constant for the reaction of BO with Alb in whole blood. We have previously described the use of Alb adducts to predict the average level of styrene-7,8-oxide in the blood of styrene-exposed workers (18).

Based on BO-Hb adducts, [BO] was calculated as follows (16):

$$[\text{BO}] = \frac{2[\text{BO} - \text{Hb}]_{\text{ss}}}{[\text{Hb}] \cdot k_{\text{BO} - \text{Hb}} \cdot t_{\text{er}}} \quad (2)$$

where $[\text{BO} - \text{Hb}]_{\text{ss}}/[\text{Hb}]$ (in pmol/g Hb) is the steady state BO-Hb adduct level, t_{er} (in h) is the zero-order elimination rate constant for the human erythrocyte, and $k_{\text{BO} - \text{Hb}}$ [in l/g Hb/h] is the second-order rate constant for the reaction of BO with Hb in whole blood. We recently measured BO in the blood of rats dosed with benzene (400 mg/kg) (27) and showed that the direct estimate of BO blood dose was similar to that predicted from BO-Alb, but was much lower than that predicted from BO-Hb (16).

Data analysis

All statistical analyses were performed using SAS system software (SAS Institute, Cary, NC) and in all cases a *P*-value of <0.05 (two-tailed) was considered significant. The precision of the overall assay and of the GC-MS measurements were estimated by performing a one-way analysis of variance (ANOVA) of the natural logarithm of the adduct level and using the error variance to calculate the coefficient of variation (CV) (28). The effect of the globin isolation technique (dialysis versus Sephadex chromatography) was also assessed using a *t*-test to compare the BO-Hb levels determined for the five samples that were isolated using both techniques.

The Wilcoxon rank sum test was used to test for differences in BO-protein adduct levels between the exposed subjects and the controls. Subjects were divided into three exposure categories [control, ≤ 31 p.p.m. (the median 8 h

TWA), and >31 p.p.m. benzene] and Spearman rank order correlation was used to correlate adduct levels with benzene exposure. To evaluate the correlation between individual adduct levels and benzene exposure, Pearson correlation coefficients were calculated. The regression of adduct levels on benzene exposure and covariates was conducted with multiple regression techniques. Two individuals had extremely high benzene exposures (>300 p.p.m.), but relatively low levels of benzene urinary metabolites (data not shown), raising a question about the exposures received by these subjects. Because these same individuals were found to have relatively low levels of BO-Hb, they were not included in any of the subsequent regression analyses of BO-Hb versus exposure and covariates. BO-Alb was not determined for these two workers.

The appropriateness of unweighted linear regression was evaluated visually from plots of the residuals (not shown). For both BO-Hb and BO-Alb, the assumption of homogeneous variance was not satisfied; hence, weighted least squares regression was used to estimate the slopes and intercepts of each adduct regressed upon benzene exposure and covariates (29). Since the true variance could not be adequately estimated, the weight was set equal to the inverse of the square of the predicted adduct level (29). After including benzene exposure in the model, the following covariates were evaluated (using stepwise variable selection techniques): age and gender (the original matching variables), respirator use, smoking status and alcohol consumption (the latter two were evaluated as both dichotomous and continuous variables).

Results

Assay variation

For BO-Hb the overall CV of the assay (including globin isolation, derivatization and GC-MS) was estimated to be 50%, based on the re-analysis of five human samples over a range of benzene exposures. Most of the variation was due to a single subject (BO-Hb = 37.9 and 158.1 pmol/g for the two determinations); if this worker was excluded, the CV was reduced to 17% ($n = 4$). The CV representing the GC-MS measurements was 10% (based on the reinjection of 32 samples). The CV of the BO-Hb assay (50%) is higher than that reported for the analysis of BO-Hb in benzene-dosed animals [~14%, reflecting derivatization and GC-MS (14)].

Recent work in our laboratory indicated that purification of rat globin by Sephadex chromatography instead of by dialysis resulted in overestimation of BO-Hb levels, potentially due to lack of removal of interferences such as glutathione conjugates (16). Ten of the globin samples in the present study had been purified by Sephadex; however, they were not found to be statistically different from the samples purified by dialysis (t -test for five samples isolated both ways; $P = 0.30$), and were therefore not treated separately in subsequent statistical analyses.

Group comparisons between BO-Hb and BO-Alb adducts and benzene exposure

Levels of BO-Hb were determined in 43 benzene-exposed workers and 42 unexposed controls, and BO-Alb was assayed in a subset consisting of 19 workers and 19 controls. Figure 1 is an example of a typical GC-MS (negative-ion chemical ionization) selected ion monitoring trace of PTTA obtained from Alb of a worker exposed to the median level (31 p.p.m.) of benzene. Summary statistics for benzene exposures and adduct levels in controls and workers exposed to ≤ 31 (the median benzene exposure as an 8 h TWA) and >31 p.p.m. benzene are given in Table I. Median adduct levels were statistically higher among all exposed workers compared with the controls (BO-Hb: 32.0 pmol/g in controls versus 79.0 pmol/g in exposed workers, $P < 0.0001$; BO-Alb: 103 pmol/g in controls versus 1220 pmol/g in exposed workers; $P < 0.0001$). When subjects were further divided into controls ($n = 42$) and workers exposed to ≤ 31 ($n = 21$) and >31 ($n = 22$) p.p.m. benzene, median BO-Hb levels were 32.0, 46.7 and

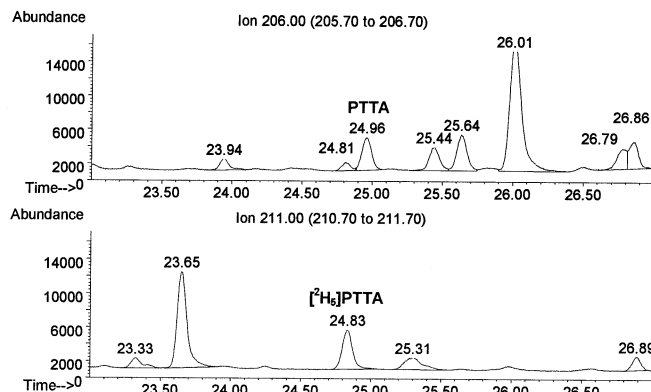


Fig. 1. Typical GC-MS (negative-ion chemical ionization) selected ion monitoring trace obtained following the reaction of Alb (4 mg) from a worker exposed to the median level of benzene (31 p.p.m. 8 h TWA) with trifluoroacetic anhydride and methanesulfonic acid to yield PTTA. [$^2\text{H}_5$]SPC (3 pmol) was added as the internal standard.

129 pmol/g globin, respectively (correlation with exposure: Spearman $r = 0.67$, $P < 0.0001$). BO-Alb levels were also highly correlated with benzene exposure, with median values in these three groups of 103 ($n = 19$), 351 ($n = 7$) and 2010 ($n = 12$) pmol/g Alb, respectively (Spearman $r = 0.90$, $P < 0.0001$). The correlation with benzene exposure for the BO-Hb levels in this same subset of workers was slightly higher than that observed for the entire cohort, with median BO-Hb levels in these three groups of 33.3 ($n = 19$), 55.6 ($n = 7$) and 141 ($n = 12$) pmol/g Hb, respectively (Spearman $r = 0.80$, $P < 0.0001$).

Individual comparisons between BO-Hb and BO-Alb adducts and benzene exposure

The relationship between BO adducts and benzene exposure was also investigated on an individual basis. The results of the weighted linear regression (after excluding the two persons identified as outliers on the basis of urinary metabolites) of adduct level on benzene exposure are given in Table II. The corresponding scatter plots of BO-Hb and BO-Alb versus benzene exposure are shown in Figure 2. The 16 workers who wore respirators for at least part of their workshift are plotted with the symbol +. After including benzene exposure in the model, weighted multiple regression was used to evaluate the impact of possible covariates on adduct levels. While age, gender, cigarettes smoked and alcohol consumption did not significantly affect levels of either BO-Hb or BO-Alb ($P > 0.1$ in all cases), respirator use was highly significant ($P \leq 0.0003$). The overall model is therefore:

$$\text{Adduct} = \beta_0 + \beta_1(\text{benzene exposure}) + \beta_2(\text{respirator use}) + \text{error}$$

with the parameter estimates and partial P -values given in Table III. If the regression of BO-Hb on benzene exposure is performed for the same subset of workers for whom BO-Alb was measured, the parameter estimates and correlation coefficients were not significantly different from those obtained using the entire cohort (<15% difference between the subset of 38 workers and the entire cohort for β_0 , β_1 and β_2).

In comparison with the estimates obtained from simple linear regression (Table II), it can be seen that controlling for respirator use (Table III) results in an ~40% reduction in the parameter estimates (β_1) for the relationship between either BO-Hb or BO-Alb and benzene exposure. These parameter estimates (after controlling for respirator use; β_1 from Table

Table I. Summary data for benzene exposures, BO-Hb and BO-Alb adducts in controls, lower-exposed workers (≤ 31 p.p.m. benzene) and higher-exposed workers (>31 p.p.m. benzene)

Parameter		Controls	Lower exposure (≤ 31 p.p.m.)	Higher exposure (>31 p.p.m.)
Benzene exposure (8 h TWA, p.p.m.)	Mean $\pm$ SD	0.015 $\pm$ 0.018	14.5 $\pm$ 9.0	109 $\pm$ 73.0
	Median (range)	0.016 (0–0.11)	13.6 (1.65–30.6)	92.0 (31.5–329)
	No. workers	44	22	22
	No. resp. users	0	1	15
	No. smokers	21	12	9
BO-Hb (pmol/g Hb)	Mean $\pm$ SD	34.2 $\pm$ 10.4	51.6 $\pm$ 24.1	136 $\pm$ 58.3
	Median (range)	32.0 (20.6–71.1)	46.7 (21.9–111)	129 (25.3–241)
	No. workers	42	21	22
	No. resp. users	0	1	15
	No. smokers	19	11	9
BO-Alb (pmol/g Alb)	Mean $\pm$ SD	107 $\pm$ 24.4	538 $\pm$ 415	2060 $\pm$ 1140
	Median (range)	103 (69.3–160)	351 (238–1220)	2010 (339–3780)
	No. workers	19	7	12
	No. resp. users	0	1	8
	No. smokers	6	2	3

Table II. Weighted^a linear regression of BO-Hb and BO-Alb adducts on benzene exposure

Adduct	<i>n</i>	β_0^b (SE)	β_1^c (SE)	<i>R</i> ²	<i>P</i> -value
BO-Hb	83 ^d	35.0 (2.2)	1.25 (0.13)	0.516	0.0001
BO-Alb	38	135 (39)	25.4 (3.4)	0.605	0.0001

^aThe variance of both Hb and Alb adducts was non-homogeneous; the adduct levels were therefore weighted by the inverse of the square of the predicted adduct level (29).
^b β_0 is the intercept, with units of pmol adduct/g protein.
^c β_1 is slope of the linear regression, with units of pmol adduct/g protein/p.p.m. benzene.
^dThe two workers identified as outliers on the basis of urinary metabolites were excluded from the regression analysis.

III) are illustrated graphically as the solid lines in Figure 2. Unexpectedly, the parameter estimate for respirator use (β_2) was positive, implying that the workers who wore respirators had higher adduct levels (after controlling for benzene exposure) than those who did not.

Background BO adducts

As shown in Table I, significant levels of background adducts were observed in the Hb and Alb of unexposed controls [BO-Hb = 34.2 $\pm$ 10.4 (SD) pmol/g, *n* = 42; BO-Alb = 107 $\pm$ 24.4 pmol/g, *n* = 19]. To gain insight into the possible origin(s) of these background adducts, we analyzed human Hb and Alb obtained from Sigma, as well as the recombinant human proteins, rHb0.1 and rHA, for BO adducts. The levels of background adducts in unexposed control workers, Sigma proteins, and recombinant proteins are summarized in Table IV. The Sigma proteins had been isolated from blood pooled from presumably unexposed individuals and contained similar levels of BO adducts to the Chinese controls (BO-Hb = 45.4 $\pm$ 8.2 pmol/g, *n* = 5; BO-Alb = 86.9 $\pm$ 31 pmol/g, *n* = 4). Significantly lower levels of both BO-Hb (19.7 $\pm$ 5.8 pmol/g, *n* = 4) and BO-Alb (41.9 $\pm$ 2.4 pmol/g, *n* = 4) were detected in the recombinant proteins.

Discussion

Bechtold *et al.* (13,15) had previously reported a significant correlation of BO-Alb, but not BO-Hb with benzene exposure in 12 exposed workers and nine controls. We report herein,

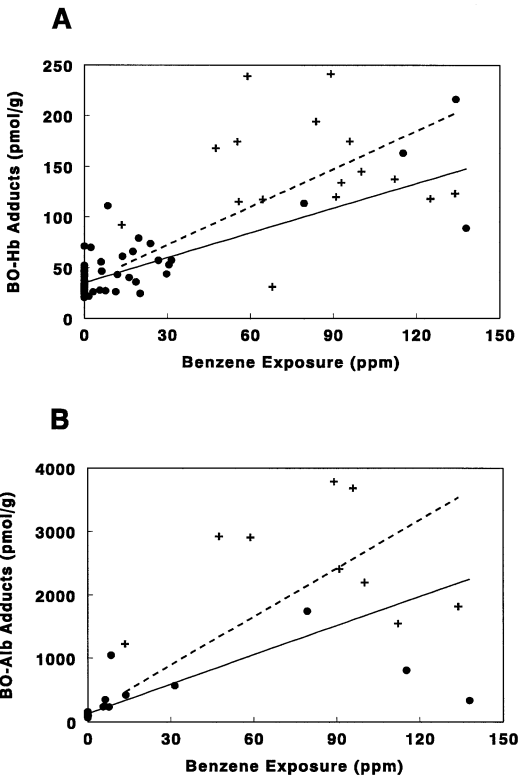


Fig. 2. Weighted least squares regression of (A) BO-Hb adducts and (B) BO-Alb adducts on benzene exposure (p.p.m., 8 h TWA). The 16 workers who wore respirators during at least part of their workshift are indicated with the symbol +. Non-respirator wearers are represented by the symbol ●. The dashed lines represent the parameter estimate for the linear regression of adduct levels on benzene exposure (β_1 of Table II), while the solid lines represent the parameter estimate of adduct levels on benzene exposure after controlling for respirator use (β_1 of Table III).

for the first time, that BO-Hb as well as BO-Alb are significantly correlated with benzene exposure (with *R*² of 0.5–0.6) in heavily exposed workers. These results indicate that BO is available for binding to blood proteins in humans exposed to benzene and that both BO-Hb and BO-Alb may be useful as biomarkers of benzene exposure, at least in highly exposed individuals.

Table III. Weighted^a multiple regression of BO-Hb and BO-Alb adducts on benzene exposure

Adduct	<i>n</i>	β_0 (SE)	β_1 (SE)	Partial R^2 (<i>P</i> -value)	β_2 (SE)	Partial R^2 (<i>P</i> -value)
BO-Hb	83 ^b	35.3 (2.0)	0.815 (0.17)	0.516 (0.0001)	52.7 (14)	0.073 (0.0003)
BO-Alb	38	130 (30)	15.4 (3.2)	0.605 (0.0001)	1160 (220)	0.172 (0.0001)

^aThe variance of both Hb and Alb adducts was non-homogeneous; the adduct levels were therefore weighted by the inverse of the square of the predicted adduct level (29). Age, gender, respirator use, smoking status and alcohol consumption were evaluated as covariates using stepwise variable selection techniques. In addition to benzene exposure, only respirator use was identified as having a significant impact on adduct levels. The overall model for both adducts therefore is: Adducts = $\beta_0 + \beta_1(\text{benzene exposure}) + \beta_2(\text{respirator use}) + \text{error}$.

^bThe two workers identified as outliers on the basis of urinary metabolites were excluded from the regression analysis.

Table IV. Background levels of BO-Hb and BO-Alb detected in control workers, Sigma proteins and recombinant proteins

Protein	BO-Hb (pmol/g Hb) mean $\pm$ SD (<i>n</i>)	BO-Alb (pmol/g Alb) mean $\pm$ SD (<i>n</i>)
Control worker	34.2 $\pm$ 10.4 (42)	107 $\pm$ 24 (19)
Sigma	45.4 $\pm$ 8.2 (5)	86.9 $\pm$ 31 (4)
Recombinant	19.7 $\pm$ 5.8 (4)	41.9 $\pm$ 2.4 (4)

Comparison with other reported BO adducts

Bechtold *et al.* (13) reported an exposure-related increase in BO-Alb in 12 occupationally exposed individuals (estimated mean exposure of 13 p.p.m.; range = 4.4–23 p.p.m. benzene), with a regression coefficient of 44 ± 8 (SE) pmol BO-Alb/g Alb/p.p.m. benzene, which is 2.9-fold higher than the 15.4 ± 3.2 pmol BO-Alb/g Alb/p.p.m. benzene found in this study. Recent evidence from our laboratory (16) indicated that the failure to dialyse protein samples may result in a ~2-fold overestimation of BO-Alb levels. If the levels of BO-Alb reported by Bechtold *et al.* (15) do reflect this overestimate, then the regression coefficients of BO-Alb levels/p.p.m. benzene exposure for these two studies are in good agreement.

Bechtold *et al.* were unable to detect BO-Hb among the same 12 workers for which they observed a dose-related increase in BO-Alb (13,15). This inability to detect BO-Hb adducts probably reflects inadequate sensitivity [500 pmol/g Hb versus 20 pmol/g Hb for the method used herein (14)].

Evaluation of covariates on BO-protein adduct levels

In addition to benzene exposure, the only covariate that had a significant impact on BO adduct levels was respirator use (Table III). Age, gender, alcohol consumption and smoking status did not significantly contribute to the model. Controlling for respirator use resulted in a reduction of ~40% in the parameter estimates for the relationship between adduct levels and benzene exposure (β_1 in Table II versus Table III). Since we were interested only in the impact of benzene exposure on BO adduct production, the values from Table III [i.e. 0.815 pmol adduct/g Hb/p.p.m. benzene for BO-Hb and 15.4 pmol adduct/g Alb/p.p.m. benzene for BO-Alb] were used for all further calculations.

Surprisingly, both BO-Hb and BO-Alb adducts were greater than proportional to benzene exposure for the 16 individuals who used respirators. Such a result is counterintuitive and implies that respirator use may be a surrogate for some other factor such as dermal exposure. In general, only those who were exposed to high levels of benzene wore respirators, and all were employed in a single workplace (Factory 1). Clearly, more work is required to elucidate the reason for this apparent anomaly.

Given that non-occupationally exposed smokers receive most of their benzene from cigarettes (estimated exposures in the USA are 0.2 mg/day for non-smokers and 2 mg/day for smokers) (30), we speculated that cigarette smoking would have a detectable impact on BO adduct levels. However, considering that a typical worker in this cohort was exposed to 31 p.p.m. $\times$ 3.2 mg/m³/p.p.m. $\times$ 1 m³/h (respiratory rate for a healthy worker engaged in light/moderate exercise) $\times$ 8 h/day = 800 mg benzene/day, it is not surprising that cigarette smoking did not have an obvious impact on the adduct levels observed in this study.

Levels of BO-Hb versus BO-Alb

Assuming that BO-protein adducts are stable and do not affect protein turnover, then the steady state levels of BO adducts are expected to be a function of both the intrinsic reactivity of BO for cysteinyl residues in Hb and Alb, and the elimination rate of the adduct. Recent studies of the kinetics of the reaction of BO with the cysteinyl residues of human Alb and Hb *in vitro* showed that the second-order rate constant for human Alb was 34.2 l/mol-h, while that for Hb was ~30-fold lower (1.16 l/mol-h), indicating that the intrinsic reactivity of BO was much greater toward serum Alb than Hb in humans (16). Coupled with the fact that the lifespan of human red blood cells (and hence of Hb) is 120–126 days (31) and that the half-life of human Alb is ~20 days (32), we expected that the levels of BO-Alb per g of protein in exposed workers would be ~14 times higher than the corresponding BO-Hb levels (16). This 14-fold difference in the expected levels of BO-Alb versus BO-Hb levels is similar to the observed difference based on the ratio of the point estimates of β_1 given in Table III (i.e. $15.4/0.815 = 19$). The relative abundance of BO-Alb compared with BO-Hb at a given level of exposure suggests that BO-Alb may be a better biomarker of human exposure to benzene.

Average blood concentration of BO

The average blood concentration of BO $[\overline{\text{BO}}]$ can be predicted from either BO-Alb adducts (equation 1) or BO-Hb adducts (equation 2). Using equation 1, with $k_{\text{Alb}} = 1.4 \times 10^{-3}/\text{h}$ (32), $k_{\text{BO-Alb}} = 5.19 \times 10^{-4}$ l/g Alb/h (16), and $[\text{BO-Alb}]_{\text{ss}}/[\text{Alb}] = 15.4$ pmol/g Alb/p.p.m. benzene (from Table III), we predict that $[\text{BO}] = 4.2 \pm 1.1$ (SE) $\times 10^{-11}$ M = 3.9 ng/l/p.p.m. benzene exposure for a typical worker in this cohort. The corresponding calculation for BO-Hb adducts (equation 2), with $t_{\text{er}} = 120$ days = 2880 h (31) and $k_{\text{BO-Hb}} = 0.177 \times 10^{-4}$ l/g Hb/h (16), and with $[\text{BO-Hb}]_{\text{ss}}/[\text{Hb}] = 0.815$ pmol/g Hb/p.p.m. benzene (from Table III) leads to an estimate of $[\text{BO}] = 3.2 \pm 0.68$ (SE) $\times 10^{-11}$ M = 3.0 ng/l/p.p.m. benzene exposure for a typical worker. A comparison of these two estimates of $[\text{BO}]$, based on BO-Alb and BO-Hb, shows that

they are not significantly different (two-sample z-test, $P = 0.43$). For a worker exposed to the median level of 31 p.p.m. benzene, we would predict that the expected average blood concentration of BO would be either 93 or 120 ng/l blood, based on BO-Hb or BO-Alb adducts, respectively.

Background BO adducts

In the control subjects, we detected surprisingly high levels of BO-Hb (mean for 42 subjects = 34.2 pmol/g) and BO-Alb (mean for 19 subjects = 107 pmol/g; Table IV). Bechtold *et al.* (15) detected background Alb adducts in two out of nine workers (up to 600 pmol/g Alb), but did not detect any Hb adducts in controls or exposed workers (limit of detection = 500 pmol/g). Background adducts of BO-Hb and BO-Alb have also been reported in rats and mice (13–16). Given that we expect (based on the linear regression of adducts on benzene exposure, Table III) 0.815 pmol BO-Hb/g Hb/p.p.m. benzene and 15.4 pmol BO-Alb/g Alb/p.p.m. benzene exposure, levels of background adducts of this magnitude would suggest unknown exposures of 7–42 [e.g. 107 pmol BO-Alb/g Alb/15.4 pmol BO-Alb/g Alb/p.p.m. = 7] p.p.m. benzene in the controls, which would result in the inhalation of 45–270 mg benzene/day [e.g. 7 p.p.m. $\times$ 3.2 mg/m³/p.p.m. $\times$ 0.5 m³/h (respiratory rate at rest) $\times$ 0.5 (uptake (33)) $\times$ 8 h/day = 45 mg benzene/day]. Such levels are clearly unrealistic given that non-occupational exposures in the USA are reported to be on the order of 2–10 p.p.b. benzene (30). Even smokers consuming 32 cigarettes/day are estimated to inhale only ~2 mg benzene/day (30). These background adduct levels were, therefore, high enough to obscure any difference between smokers and non-smokers in the control population. For BO-Hb, smokers had an average adduct level of 33.5 ± 8.5 (SD) pmol/g ($n = 19$) while non-smokers had 34.8 ± 12 pmol/g ($n = 23$). For BO-Alb, the levels were 94.1 ± 19 pmol/g ($n = 6$) for smokers and 113 ± 25 pmol/g ($n = 13$) for non-smokers.

The detection of such high levels of background adducts points to a number of potential explanations, including the possibility that our assay artifactually overestimates the adduct level, that the control workers were exposed to a compound other than benzene (such as BO itself) that also gives rise to BO adducts or that there is a mechanism that leads to the endogenous production of adducts [such as lipid peroxidation or metabolism by intestinal gut flora, which have been implicated in the formation of background ethylene oxide adducts (19)]. To investigate these high levels of BO adducts, we compared the levels in the proteins of Chinese controls with adduct levels in Hb and Alb obtained from Sigma (representing pooled proteins from presumably unexposed persons in the USA) and in recombinant human Hb (derived from *E.coli*) and Alb (derived from yeast). As shown in Table IV, the Sigma proteins and the proteins from the Chinese controls contained similar levels of BO-Hb (45.4 and 34.2 pmol/g, respectively) and BO-Alb (86.9 and 107 pmol/g, respectively), indicating that the background adducts cannot be ascribed to differences in diet or the environment between the USA and China. The recombinant proteins contained approximately half the levels of both BO-Hb (19.7 pmol/g) and BO-Alb (41.9 pmol/g) that were found in the human samples. Since yeast and bacteria do not smoke or pump gas, they are expected to be exposed to much lower levels of benzene than humans; we interpret the adducts observed in the recombinant proteins as a probable artifact of the assay. However, it is also possible that these adducts represent endogenous formation of adducts,

potentially due to a metabolic pathway common to humans, *E.coli* and yeast. The difference between the adducts detected in the recombinant proteins and those from the control workers (34.2 – 19.7 = 14.5 pmol/g for BO-Hb and 107 – 41.9 = 65.1 pmol/g for BO-Alb), and the Sigma proteins would therefore represent either unknown environmental or dietary exposures or endogenous production of adducts via a metabolic pathway unique to higher organisms. Although the interpretation of these background adducts is still unclear, we encourage others to explore the use of recombinant proteins in their investigations of background protein adducts.

Conclusions

We report, to our knowledge, the first observed correlation between levels of BO-Hb and benzene exposure in humans. As has been noted by other investigators (13), we also found a significant correlation between levels of BO-Alb and benzene exposure. Both adducts lead to similar predictions of BO in the blood of exposed workers, lending credence to the use of both BO-Hb and BO-Alb as biomarkers of benzene exposure. However, the detection of high levels of background adducts in unexposed persons and recombinant proteins may limit the utility of these markers to distinguish individuals exposed to lower levels of benzene. The biological significance of these background adducts is under investigation.

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