

Protein Adducts of 1,4-Benzoquinone and Benzene Oxide among Smokers and Nonsmokers Exposed to Benzene in China¹

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Abstract

Hemoglobin (Hb) and albumin (Alb) adducts of the benzene metabolites benzene oxide (BO) and 1,4-benzoquinone (1,4-BQ) were analyzed by gas chromatography-mass spectrometry in 43 exposed workers and 44 unexposed controls from Shanghai, China, as part of a larger cross-sectional study of benzene biomarkers. When subjects were divided into controls ($n = 44$) and workers exposed to ≤ 31 ppm ($n = 21$) and > 31 ppm ($n = 22$) of benzene, median 1,4-BQ-Alb adducts were 2110, 5850, and 13,800 pmol/g Alb, respectively (correlation with exposure: Spearman $r = 0.762$; $P < 0.0001$); median BO-Alb adducts were 106, 417, and 2400 pmol/g Alb, respectively (Spearman $r = 0.877$; $P < 0.0001$); and median BO-Hb adducts were 37.1, 50.5, and 136 pmol/g Hb, respectively (Spearman $r = 0.757$; $P < 0.0001$). To our knowledge, this is the first observation that adducts of 1,4-BQ are significantly correlated with benzene exposure. When compared on an individual basis, Alb adducts of 1,4-BQ and BO and Hb adducts of BO were highly correlated with each other and with urinary phenol and hydroquinone ($P < 0.0001$ for all of the comparisons). Although detectable in the assays, Hb adducts of 1,4-BQ and both Hb and Alb adducts of 1,2-BQ produced erratic results and are not reported. Interestingly, cigarette smoking increased Alb adducts of 1,4-BQ but not of BO, suggesting that benzene

from cigarette smoke was not the primary contributor to the 1,4-BQ adducts.

Introduction

Benzene is an important industrial and environmental chemical that causes leukemia in humans and various cancers in experimental animals (1, 2). Although the mechanism of benzene carcinogenicity is unclear, it is thought to involve the action of one or more metabolites. Benzene is initially metabolized via cytochrome P4502E1 (and possibly other cytochrome P450s) to BO³, which is in equilibrium with its valence tautomer oxepin (3–5). Most of the BO-oxepin spontaneously rearranges to produce phenol, whereas smaller amounts react to produce the phenolic products HQ (after P450 oxidation of phenol) and CAT (formed via hydrolysis of BO to benzene dihydrodiol and subsequent action by dihydrodiol dehydrogenase), as well as the muconaldehydes (after a second P450 oxidation of the oxepin and subsequent ring opening; Refs. 4, 6, 7). Oxidation of HQ and CAT, either via autooxidation or myeloperoxidase, gives rise to 1,4- and 1,2-BQ, respectively (8).

The mechanism of benzene carcinogenicity has been difficult to elucidate because BO, the BQs, and the muconaldehydes are all electrophilic and capable of reacting with macromolecules such as DNA and proteins (9–11). Nonetheless, most investigators have postulated a prominent role for 1,4-BQ, because it has been shown to bind to macromolecules, to interfere with the microtubule assembly, to cause chromosomal fragmentation during cell division, and to inhibit cell division and DNA synthesis (12–14). Recent attention has also focused on BO, the primary benzene metabolite, which binds to macromolecules and is sufficiently stable (half-life of several minutes) to be distributed from the liver to other tissues (3, 15).

The potential importance of BO and 1,4-BQ in the carcinogenesis of benzene has motivated studies of biomarkers to investigate the dispositions of these metabolites after exposure to benzene. However, because BO and 1,4-BQ are themselves reactive and, therefore, difficult to measure, traditional bio-monitoring involved measurement of the stable metabolites (*i.e.*, phenol, HQ, and their sulfate and glucuronide conjugates) in blood and urine (16–18). More recently, cysteinyl adducts of BO and 1,4-BQ with Hb and/or plasma Alb have been used for additional investigations (19–21). (Henceforth, the BO and 1,4-BQ adducts will be designated BO-Hb, BO-Alb, 1,4-BQ-Hb, and 1,4-BQ-Alb).

We reported previously the relationships between benzene exposure and levels of BO-Hb, BO-Alb, 1,4-BQ-Hb, and 1,4-

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³ The abbreviations used are: BO, benzene oxide; Alb, albumin; BQ, benzoquinone; CAT, catechol; CV, coefficient of variation; GC-MS, gas chromatography-mass spectrometry; Hb, hemoglobin; HQ, hydroquinone; HQ-S-TFA, O,O',S-tris-trifluoroacetyl-hydroquinone; ICC, interclass correlation; SPC, S-phenylcysteine; TWA, time-weighted average.

BQ-Alb in rats and mice dosed with labeled benzene (21–24). More recently, we provided analogous relationships for BO-Hb and BO-Alb among benzene-exposed workers and controls in Shanghai, China (BO-Alb adducts were only analyzed in a subset of subjects; 1,4-BQ adducts had not been analyzed previously; Ref. 19). The purpose of the current study is to report levels of BO-Hb, BO-Alb, and 1,4-BQ-Alb among the full complement of these workers (44 currently exposed to benzene and 44 controls) based on reanalysis of proteins isolated previously. In doing so, we will correlate levels of BO-Hb, BO-Alb, and 1,4-BQ-Alb with personal benzene exposures, as well as with levels of urinary benzene, phenol, and HQ that were reported separately (25, 26). (Although 1,4-BQ-Hb was also detected in the assays, levels were indistinguishable from control values, and results were erratic; thus, these data will not be presented). Because we ultimately wish to extend the exposure-biomarker relationships to lower levels of benzene exposure, we will also explore the contribution of cigarette smoking, a known source of benzene and HQ, on the background levels of the protein adducts of BO and 1,4-BQ.

Materials and Methods

Reagents. Human Hb and Alb were obtained from Sigma Chemical Co. (St. Louis, MO). [$^2\text{H}_5$]SPC was synthesized as described previously (19). Methanesulfonic acid was purchased from Fluka Chemical Co. (Buchs, 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 by gender and age (5-year intervals). Each subject was administered a questionnaire to collect data on age, gender, current and lifelong tobacco use, alcohol consumption, medical history, and work history (27).

Here we report Alb adducts of 1,4-BQ for 43 (of 44) exposed and 43 (of 44) unexposed workers, which were measured on two separate occasions in the current study. In addition, we reassayed BO-Hb and BO-Alb among the same subjects and compared results from the first assay, reported previously (19) with the current assay.

Collection of Air and Biological Samples. Individual exposures were monitored using passive personal monitors worn by each exposed worker during the full work shift on 5 consecutive days during the 1–2 week period before blood collection (27). The geometric mean of the five air measurements was used to estimate the individual full-shift exposure to benzene in ppm (note that 1 ppm = 3.2 mg benzene/m³). It should be noted that 16 workers in factory 1 used half-mask charcoal respirators during the highest exposure period of their work shift, which lasted ~3 h. Overall, the workers were exposed to a median full shift level of 31 ppm of benzene (27).

Subjects in the control factory (where sewing machines were manufactured) were monitored for benzene exposure on 1 day of 6 different sampling days. Subjects in the administrative facility were assumed to have no exposure to benzene (27).

Exposed workers (43 of 44) provided a spot urine sample at the end of a work shift or at the end of the high exposure period of their work shift (factory 1). Urine from the unexposed workers was obtained at a clinic. Venous blood (27 ml) was obtained from 43 exposed and 44 unexposed workers toward the end of the work shift.

Protein-Adduct Analysis. Because 1,4-BQ adducts can exist in the oxidized (quinone) form after formation, they can undergo additional substitution reactions on the aromatic ring until all four of the positions have been filled (28). We measured only the mono-S-substituted 1,4-BQ adducts of Hb and Alb using the procedure developed for BO-Hb (24) and extended to BQ adducts with minor modifications (21).

After isolation, 4–5 mg of Alb or 10 mg of Hb, 20 μg of [$^2\text{H}_5$]1,4-BQ-Alb or -Hb, and 10 pmol of [$^2\text{H}_3$]SPC (internal standards for the 1,4-BQ and BO adducts, respectively), and 200 μl of 100-mM ascorbic acid (to convert the adducts to the HQ form before trifluoroacetylation) were dried in a vacuum oven. Trifluoroacetic anhydride (800 μl) and 20 μl of 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 of 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 GC-MS in the negative-ion chemical-ionization mode using selected ion monitoring for the derivatized analyte, HQ-S-TFA (m/z 333), and the derivatized internal standard ([$^2\text{H}_3$]HQ-S-TFA, m/z 336). The BO adducts were analyzed in the same samples during a subsequent GC-MS injection as described in Yeowell-O'Connell *et al.* (19).

It is also possible to detect the protein adducts of 1,2-BQ (O,O',S-tris-trifluoroacetyl-CAT, m/z 333; Ref. 21). However, these derivatized adducts are very unstable after washing and, given our lack of a chemically equivalent internal standard, could not be reproducibly analyzed. The Hb adducts of 1,4-BQ, in sharp contrast to those of Alb, also gave irreproducible results. We are currently investigating the addition of an iron chelator to the reaction to stabilize the Hb adducts.

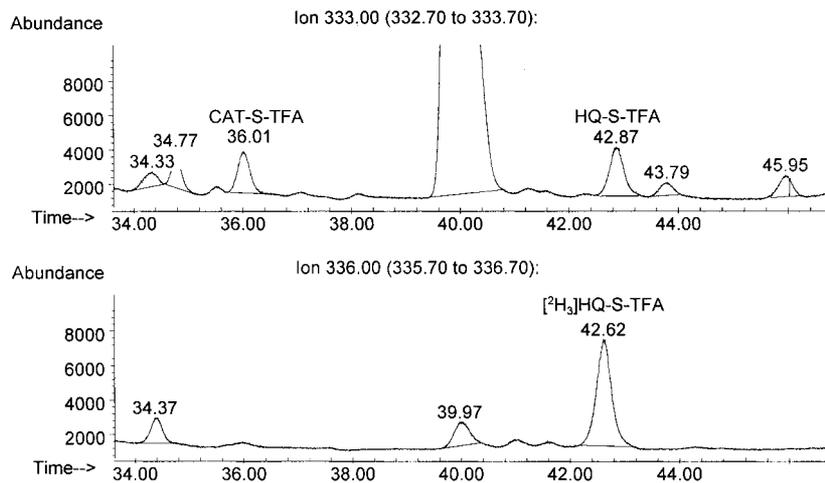
Urinary Metabolites. Previously reported levels of urinary benzene (μg /liter urine) were obtained for 83 (41 control and 42 exposed) workers (26), and levels of urinary phenol and HQ (μg /mg creatinine) were obtained for 60 (17 control and 43 exposed) workers (25).

Measures of Cigarette Consumption. Subjects reported the average number of cigarettes smoked per day during the past month via questionnaire (median = 10 cigarettes/day, range = 4–20). Urinary cotinine (μg /mg creatinine) was measured by GC-MS (27).

Data Analysis. All of the statistical analyses were performed using SAS system software (SAS Institute Inc., Cary, NC), and in all cases a P of <0.05 (two-tailed) was considered significant. Two individuals having extremely high benzene exposures (>300 ppm) but relatively low levels of urinary benzene and benzene metabolites were excluded from the analyses as outliers (19, 26). In addition, one exposed worker did not provide sufficient blood for analysis, and one 1,4-BQ-Alb sample was lost during processing.

The precision of assays for measurement of the protein adducts as well as the GC-MS injections was estimated from the variance components obtained with a nested random effects model (injections nested within assays) via Proc NESTED of SAS. The data for these analyses were obtained by randomly reinjecting 25% of a subset of the samples. Because the data

Fig. 1. Typical GC-MS (negative ion chemical ionization)-selected ion-monitoring trace obtained after the reaction of Alb (4 mg from a benzene-exposed worker) with trifluoroacetic anhydride and methanesulfonic acid to yield HQ-S-TFA. [$^2\text{H}_5$]1,4-BQ-Alb (20 μg) was added as the internal standard.



were skewed and displayed obvious heteroscedasticity, the random effects model was performed using the natural logarithms of the adduct levels. Precision is reported in terms of $\text{CV} = [\exp(s^2)-1]^{1/2}$, assuming lognormally distributed data (29), where s^2 represents the estimated variance component for either the GC-MS injection (the error variance component) or the assay (the between-group variance component).

Agreement between pairs of assay results for BO-Hb, BO-Alb, and 1,4-BQ-Alb conducted 16–19 months apart was assessed in terms of the ICC after application of a one-way random effects model via Proc NESTED of SAS.

The Wilcoxon rank-sum test was used to evaluate differences in protein adduct levels between the exposed subjects and the controls. Correlation of levels of benzene exposure, urinary biomarkers, protein adducts, and cigarette consumption (urinary cotinine) was evaluated on both a group and an individual basis using Spearman coefficients. For the group correlations, subjects were divided into three exposure categories [control, 61–31 ppm (the median full-shift exposure), and >31 ppm benzene]. For individual correlations, subject-specific geometric means of exposure measurements (from 5 consecutive work-days before blood collection) were used along with subject-specific means of replicate biomarker measurements.

Because scatter plots of adduct levels *versus* individual exposure displayed obvious heteroscedasticity, weighted multiple linear regression was used to investigate relationships between the adduct levels and benzene exposures of individuals after controlling for significant covariates. Because the true variance could not be adequately estimated, weights were set equal to the inverse of the square of the predicted adduct levels (30). After including benzene exposure in the model, the following covariates were evaluated: age and gender (the original matching variables), respirator use, smoking status (evaluated as smokers *versus* nonsmokers, number of cigarettes smoked per day, and urinary cotinine levels), and alcohol consumption (evaluated as both a dichotomous and continuous variable). Variables were chosen for inclusion in a model using forward stepwise selection at a significance level of 0.15. After extensive preliminary evaluation of all of the possible covariates, final models were constructed including exposure and respirator use for all of the adducts and also urinary cotinine for 1,4-BQ-Alb but not for BO-Alb and BO-Hb. Although urinary cotinine was selected for the final model, generally consistent results were obtained using smoking/nonsmoking or cigarettes/day as measures of cigarette consumption.

Table 1 CVs for replicate GC-MS injections and assays

Adduct	Injection CV (n^a)	Assay CV (n^a)
1,4-BQ-Alb	11% (50)	39% (54)
BO-Alb	8.4% (49)	36% (48)
BO-Hb	10% (60)	28% (116)

^a 1–3 replicates were analyzed per sample.

Table 2 Agreement (given as the ICC) of replicate assays of protein adducts analyzed at different times

Adduct	ICC (n^a)	Time interval (mo)
1,4-BQ-Alb	0.95 (38)	16–17
BO-Alb	0.96 (40)	17–18
BO-Hb	0.87 (85)	18–19

^a No. of data pairs.

Results

GC-MS of 1,4-BQ-Alb. Fig. 1 shows of a typical GC-MS (negative ion chemical ionization)-selected ion monitoring trace of 1,4-BQ-Alb (the derivatized product is referred to as HQ-S-TFA) obtained from a worker exposed to the median level (31 ppm) of benzene. The limit of detection (signal-to-noise ratio of 3:1) for 1,4-BQ-Alb is the same as that reported for the BO adduct (20 pmol/g; Refs. 21, 24). However, the lowest level of 1,4-BQ-Alb detected in unexposed workers was 949 pmol/g, well above the limit of detection and much higher than the observed levels of BO-Alb and BO-Hb.

Assay Precision and Agreement. As shown in Table 1, between 48 and 116 protein specimens were assayed two to four times. The CVs estimated from these replicate assays ranged between 28 and 39%. The component of variability associated with GC-MS injections corresponded to a CV of 8.4–11%.

Data pairs representing repeated assays of BO-Hb (85 subjects), BO-Alb (40 subjects), and 1,4-BQ-Alb (38 subjects) conducted 16–19 months apart were analyzed with a random effects model. The results, summarized in Table 2, indicate very good agreement, with ICCs in the range of 0.87–0.96 (an ICC of 1 would indicate perfect agreement). This indicates that purified proteins can be stored for long periods at -80°C without compromising the integrity of the samples.

Table 3 Summary statistics for benzene exposures, 1,4-BQ-Alb, BO-Alb, and BO-Hb, in controls, low-exposed workers (≤ 31 ppm benzene) and high-exposed workers (> 31 ppm benzene)

Parameter		Controls (<i>n</i> = 44)	≤ 31 ppm benzene (<i>n</i> = 21)	> 31 ppm benzene (<i>n</i> = 22)
Individual exposure (ppm)	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 (31.5–328)
1,4-BQ-Alb (pmol/g Alb)	Mean \pm SD	2,730 \pm 1,680	6,330 \pm 3,090	15,000 \pm 9,630
	Median (Range)	2,110 (949–9,410)	5,850 (2,410–15,020)	13,800 (3,200–38,800)
BO-Alb (pmol/g Alb)	Mean \pm SD	122 \pm 54.2	548 \pm 413	2,380 \pm 1,340
	Median (Range)	106 (6.9–248)	417 (160–1,520)	2,400 (323–5,770)
BO-Hb (pmol/g Hb)	Mean \pm SD	37.8 \pm 9.1	56.2 \pm 27.6	144 \pm 53.2
	Median (Range)	37.1 (23.3–62.5)	50.5 (27.4–157)	136 (30.9–245)

Table 4 Correlation matrix for exposure, protein adducts, and urinary biomarkers from all subjects^a

	Group Exposure	BO-Alb	1,4-BQ-ALb	BO-Hb	Urinary benzene	Urinary phenol	Urinary HQ	Urinary cotinine
Individual exposure	0.933	0.833	0.782	0.718	0.810	0.832	0.870	0.056
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.607
Group exposure	86	85	84	85	81	58	58	86
		0.875	0.770	0.729	0.879	0.839	0.882	–0.029
BO-Alb		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.790
		87	86	87	83	60	60	88
1,4-BQ-Alb			0.740	0.767	0.758	0.783	0.839	–0.126
			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.244
BO-Hb			86	87	82	59	59	87
				0.618	0.639	0.589	0.690	0.242
Urinary benzene				<0.0001	<0.0001	<0.0001	<0.0001	0.025
				86	81	58	58	86
Urinary phenol					0.643	0.757	0.777	–0.046
					<0.0001	<0.0001	<0.0001	0.674
Urinary HQ					82	59	59	87
						0.831	0.875	–0.091
						<0.0001	<0.0001	0.412
						57	57	83
							0.943	–0.101
							<0.0001	0.443
							60	60
								–0.082
								0.533
								60

^a Spearman correlation, *P*, *n* shown for each.

Levels of 1,4-BQ-Hb, 1,2-BQ-Hb, and 1,2-BQ-Alb gave irreproducible results and are not reported.

Group Comparisons between Protein Adducts and Benzene Exposure. Median adduct levels were significantly higher among all of the exposed workers compared with the controls (1,4-BQ-Alb: 2110 pmol/g in controls *versus* 7990 pmol/g in exposed workers, $P < 0.0001$; BO-Alb: 106 pmol/g in controls *versus* 806 pmol/g in exposed workers, $P < 0.0001$; BO-Hb: 37.1 pmol/g in controls *versus* 77.2 pmol/g in exposed workers, $P < 0.0001$). As shown in Table 3, when subjects were additionally divided into controls ($n = 44$) and workers exposed to ≤ 31 ppm ($n = 21$) and > 31 ppm ($n = 22$) benzene, levels of 1,4-BQ-Alb, BO-Alb, and BO-Hb all increased with the category of benzene exposure. The Spearman correlation coefficients for these trends were 0.762 for 1,4-BQ-Alb, 0.877 for BO-Alb, and 0.757 for BO-Hb, all with $P_s < 0.0001$.

Correlation of Protein Adducts, Benzene Exposure, Urinary Biomarkers, and Cigarette Smoking. Table 4 provides the correlation matrix relating benzene exposure (individual and grouped) with the protein adducts (1,4-BQ-Alb, BO-Alb, and BO-Hb), the urinary biomarkers (benzene, phenol, and

HQ), and cigarette smoking (as urinary cotinine). The Spearman coefficients indicate that all of the protein and urinary biomarkers were highly correlated ($P < 0.0001$) with individual benzene exposure and with each other. (Although not shown, similar Spearman coefficients were also observed for urinary CAT and muconic acid). Of the other covariates, respirator use was positively correlated with both individual exposure and the benzene biomarkers ($P < 0.0001$), whereas gender and alcohol consumption were not correlated with either exposure or the biomarkers.

Impact of Cigarette Smoking on Benzene Biomarkers. The measure of cigarette smoking, urinary cotinine, was significantly correlated with 1,4-BQ-Alb ($P = 0.025$) but not with the BO adducts or with the urinary biomarkers of benzene. This relationship between adducts and cigarette smoking is additionally explored in Fig. 2, A and B, which clearly delineates the additive effects of grouped benzene exposure ($P < 0.0001$ for both 1,4-BQ-Alb and BO-Alb) and cigarette smoking on production of 1,4-BQ-Alb (Fig. 2A; $P = 0.034$) but not of BO-Alb (Fig. 2B; $P = 0.23$), shown for comparison. The number of subjects and mean levels of benzene exposure, 1,4-BQ-Alb and

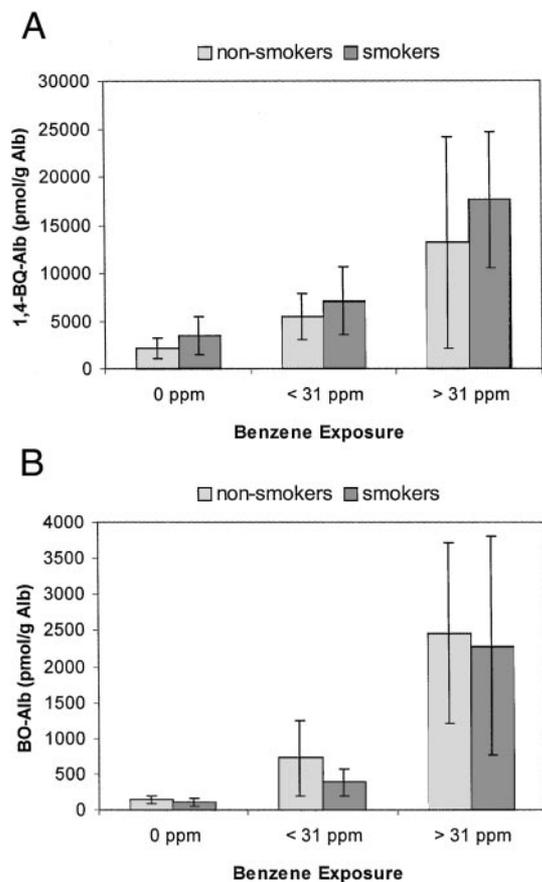


Fig. 2. A, effect of benzene exposure (unexposed, lower, and higher; $P < 0.0001$) and cigarette smoking (nonsmokers versus smokers; $P = 0.034$) on levels of 1,4-BQ-Alb. B, effect of benzene exposure (unexposed, lower, and higher; $P < 0.0001$) and lack of effect of cigarette smoking (nonsmokers versus smokers; $P = 0.23$) on levels of BO-Alb. Bars, \pm SD.

BO-Alb, for smokers and nonsmokers in the three exposure categories are provided in Table 5.

Multiple Regression Analysis of Adduct Levels. Scatter plots of levels of 1,4-BQ-Alb, BO-Alb, and BO-Hb versus individual benzene exposures are shown in Fig. 3A–C. Note that the 16 respirator users are designated with a “+”.

Using stepwise procedures, multiple regression of adduct levels on individual exposure and covariates confirmed the importance of exposure and respirator use in all of the cases and of cigarette consumption (as urinary cotinine) on 1,4-BQ-Alb but not BO-Alb or BO-Hb. The effects of age, gender, and alcohol consumption on adduct levels were not significant even after controlling for benzene exposure and respirator use. Final models for the three protein adducts are summarized in Table 6. In each case, the intercept (β_0) was highly significant indicating the presence of the same adducts among unexposed subjects. Judging by the magnitudes of the partial R^2 values, benzene exposure explained between 43 and 62% of the variability of adduct levels, respirator use explained 3–15%, and cigarette consumption (urinary cotinine) explained 8% (for 1,4-BQ-Alb only). The parameter estimates for respirator use (β_2) were positive for all three of the adducts, implying that the workers who worked in factory 1 and wore respirators had higher adduct levels (after controlling for benzene exposure) than those who did not.

If the analysis is limited to only those workers exposed to <31 ppm benzene ($n = 64$), significant exposure-response relationships remain for the three adducts (1,4-BQ-Alb, $r = 0.356$, $P < 0.0001$; BO-Alb, $r = 0.512$, $P < 0.0001$; and BO-Hb, $r = 0.132$, $P = 0.003$).

Discussion

This is the first report that 1,4-BQ-Alb is significantly correlated with benzene exposure among occupationally exposed subjects, and confirms previous findings that BO adducts are also correlated with benzene exposure. This indicates that both 1,4-BQ and BO are available for binding to plasma proteins in humans exposed to benzene and that 1,4-BQ-Alb, BO-Alb, and BO-Hb can serve as biomarkers of benzene exposure, at least among highly exposed individuals. In addition, our results indicate that cigarette smoking significantly contributes to the systemic level of 1,4-BQ (but not BO) among these subjects.

Evaluation of Covariates on Protein Adduct Levels. In addition to benzene exposure, respirator use and smoking status (as urinary cotinine) also significantly contributed to the levels of 1,4-BQ-Alb, whereas age, gender, and alcohol consumption did not. Only benzene exposure and respirator use contributed to the levels of BO adducts, consistent with our earlier results (19). Surprisingly, levels of 1,4-BQ-Alb, BO-Alb, and BO-Hb were all greater than expected for the 16 individuals who used respirators (all used in factory 1). Such a result is counterintuitive and implies that respirator use may be a surrogate for some other factor specific to factory 1. These workers were exposed to very high levels of benzene for a period of 2–3 h, and there was evidence that respirator breakthrough had occurred. If exposure to very high levels of benzene for brief periods results in a greater blood dose of BO and 1,4-BQ than would be predicted from the 8-h TWA exposure, then respirator use may simply indicate that the 8-h TWA effectively underestimated the systemic doses of benzene metabolites received by these workers. Such behavior would be consistent with BO levels in the blood of rats receiving a bolus dose of 400 mg of benzene/kg of body weight, where the peak blood level of BO was maintained for ~ 10 h after administration (3). Unfortunately, because the small number of workers who wore respirators were not uniformly distributed across the sample (all worked in factory 1), it is difficult to additionally address this question. This difficulty points to the potential utility of using protein adducts to more accurately assess the doses of electrophilic metabolites of benzene among highly exposed workers.

Of particular interest is our finding that cigarette smoking significantly increased the levels of 1,4-BQ adducts but not BO adducts in these workers (Fig. 2 and Table 5). Given that the typical nonoccupationally exposed smoker receives most of his or her benzene exposure from cigarettes (estimated exposures in the United States are 0.2 mg/day for nonsmokers and 2 mg/day for smokers; Ref. 31), we anticipated a detectable impact of cigarette consumption on BO adduct levels. However, considering that a typical worker in our sample was exposed to $31 \text{ ppm} \times 3.2 \text{ mg/m}^3 (\text{ppm})^{-1} \times 1 \text{ m}^3/\text{h}$ (respiratory rate for a healthy worker engaged in light/moderate exercise $\times 8 \text{ h/day} = 800 \text{ mg benzene/day}$), it is perhaps not surprising that cigarette smoking would not have a detectable effect on the regression of BO adducts on exposure. Nonetheless, our data provide no evidence of a smoking-related increase in BO adducts even among control subjects (additionally discussed below) and, thus, suggests that the effect of smoking on levels of 1,4-BQ-Alb was not attributable to benzene in cigarette smoke. This conclusion is consistent with measurements

Table 5 Mean benzene exposure, 1,4-BQ-Alb and BO-Alb levels for smokers and nonsmokers in three exposure categories

Exposure category	Smoking category	n	Benzene exposure (ppm)		1,4-BQ-Alb (pmol/g)		BO-Alb (pmol/g)	
			Mean	SD	Mean	SD	Mean	SD
control	nonsmokers	23	0.010	0.012	2096	1070	136	53
control	smokers	20	0.021	0.022	3469	1967	108	53
≤31 ppm	nonsmokers	10	14.05	10.03	5467	2346	723	526
≤31 ppm	smokers	11	14.93	8.39	7116	3573	390	187
>31 ppm	nonsmokers	12	83.89	31.54	13196	11000	2457	1256
>31 ppm	smokers	8	95.72	31.39	17607	7015	2277	1521

of urinary benzene among the same workers, where no difference was detected between smoking and nonsmoking control subjects (26).

Background Adducts. In the control subjects (Table 3, $n = 44$), we detected high levels of both 1,4-BQ-Alb (mean 1,4-BQ-Alb = 2730 pmol/g) and BO adducts (mean BO-Alb = 122 pmol/g and BO-Hb = 37.8 pmol/g). As we suggested previously (19), it is unrealistic to suppose that such background levels could have arisen from benzene exposure, given the exposure-related regression coefficients observed (values of β_1 in Table 6). For example, based on $\beta_1 = 124$ pmol of 1,4-BQ-Alb/g Alb/ppm of benzene exposure, a control subject in our sample would have required exposure to 22 ppm of benzene 8 h/day for 5 days/week to produce the observed background adducts. This is clearly unrealistic based on nonoccupational exposures in the range of 2–10 ppb of benzene (31). Equivalent calculations using control levels of BO-Alb and BO-Hb lead to similarly unrealistic predictions of background benzene exposure of 6 and 42 ppm, respectively. We also note that a similar level of background BO-Hb (29 pmol/g) was reported recently by investigators using an entirely different method of analysis (32). This implies that these background BO adducts represent exposure to an endogenous or exogenous substance that results in formation of SPC, the adduct detected in both assays.

However, in contrast to the BO adducts, which have no known background sources, 1,4-BQ adducts could arise from non-benzene-derived HQ via the metabolism of phenol, a product of gut metabolism (33) and arbutin, the glucose conjugate of HQ, which has been identified in a variety of foods (17). In addition, HQ has been detected in cigarette smoke (34). Detectable levels of HQ have been observed in both urine (16, 18, 25, 35) and plasma (17) of unexposed humans, and urinary HQ has been positively correlated with cigarette smoking (35, 36). Earlier work from our laboratory also reported high levels of BQ adducts in unexposed mice, rats, and humans (9, 22, 23, 37).

As shown in Fig. 2A, smoking accounted for about half of the 1,4-BQ-Alb observed in the unexposed subjects. Fig. 4A additionally illustrates the positive association between 1,4-BQ-Alb and cigarette smoking (as urinary cotinine) in the 44 unexposed workers ($R^2 = 0.384$; $P < 0.0001$). Although we did not detect an association between urinary HQ and cigarette smoking in this study, most likely because of the limited number of urine samples from unexposed workers that were analyzed (17 of 44), we suspect that HQ from cigarette smoke contributed to this smoking-related increase in 1,4-BQ-Alb. Regarding BO-Alb, Figs. 2B and 4B both point to a (nonsignificant) negative association between BO-Alb and cigarette smoking. If real, such a negative association would suggest either decreased production or increased removal of BO among smokers exposed to high levels of benzene, possibly related to metabolic induction by cigarette smoke.

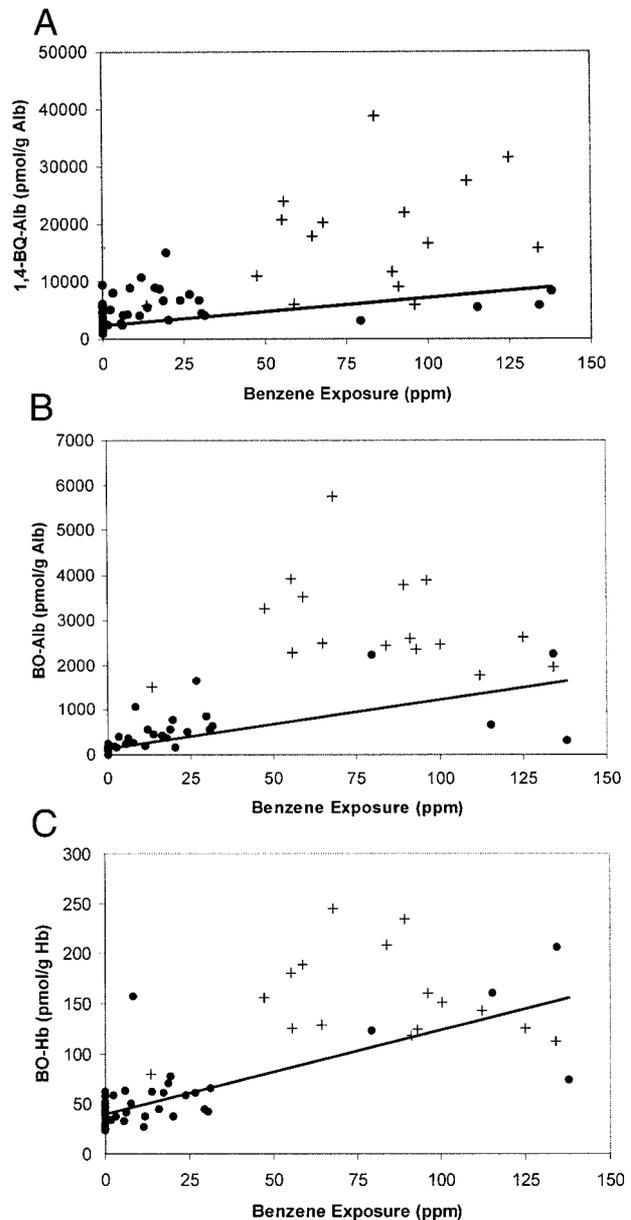


Fig. 3. Weighted least squares regression of (A) 1,4-BQ-Alb (B) BO-Alb and (C) BO-Hb adducts on benzene exposure (ppm; 8-h TWA). The 16 workers who wore respirators during at least part of their workshift are indicated with the symbol +. Nonrespirator wearers are represented by the ●. —, parameter estimate of adduct levels on benzene exposure after controlling for respirator use and smoking (β_1 of Table 6).

Table 6 Weighted multiple regression of 1,4-BQ-Alb, BO-Alb, and BO-Hb on benzene exposure, respirator use, and urinary cotinine

Table is based on the following model: adduct level (pmol/g protein) = $\beta_0 + \beta_1(\text{benzene exposure, ppm}) + \beta_2(\text{respirator use}) + \beta_3(\text{cotinine, } \mu\text{g/mg creatinine}) + \text{error}$.

Adduct	n	β_0 (SE)	β_1 (SE)	Partial R ² (P)	β_2 (SE)	Partial R ² (P)	β_3 (SE)	Partial R ² (P)
1,4-BQ-Alb	84	2360 (327)	124 (25.0)	0.426 (0.0001)	4470 (2030)	0.028 (0.030)	0.757 (0.204)	0.078 (0.0006)
BO-Alb	85	134 (16.7)	19.3 (2.60)	0.622 (0.0001)	1430 (197)	0.147 (0.0001)	NS ^a	
BO-Hb	85	39.3 (2.10)	0.860 (0.169)	0.535 (0.0001)	52.6 (14.1)	0.068 (0.0003)	NS ^a	

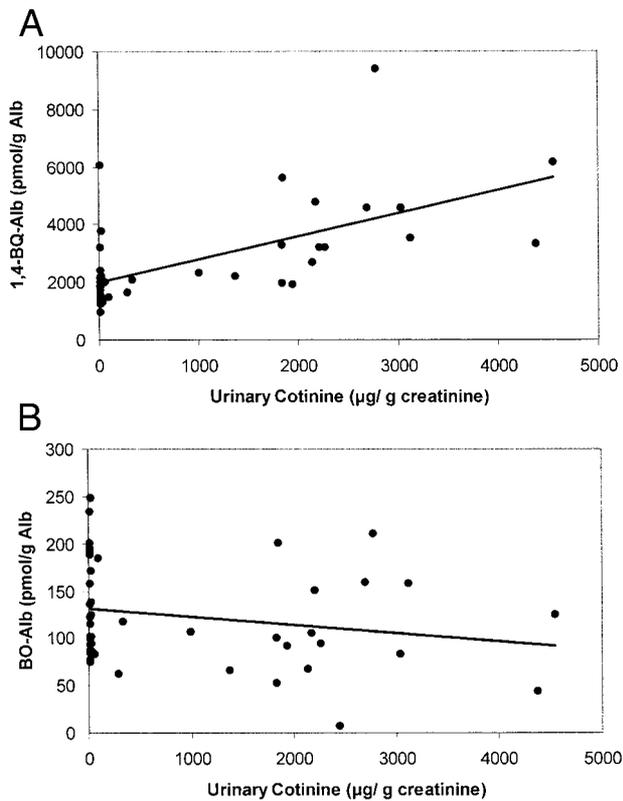
^a Effect is not sufficiently significant for inclusion in the model ($P > 0.15$).

Fig. 4. A, significant positive relationship between 1,4-BQ-Alb and cigarette smoking (as urinary cotinine) in 44 unexposed workers ($R^2 = 0.384$; $P < 0.001$). B, nonsignificant negative relationship between BO-Alb and cigarette smoking in 44 unexposed workers ($R^2 = 0.042$; $P = 0.183$).

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