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Non-linear production of benzene oxide–albumin adducts with human exposure to benzene

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Abstract

Benzene is initially metabolized to benzene oxide, which either undergoes further metabolism or reacts with macromolecules including proteins. Previously reported levels of benzene oxide–albumin adducts (BO–Alb) are analyzed from 30 workers exposed to 0.2–302 ppm benzene and 43 controls from Shanghai, China. Although both exposed workers and controls had significant levels of BO–Alb in their blood, exposed subjects’ adduct levels (GM=378 pmol/g protein) were much greater than those of controls (GM=115 pmol/g protein). When the natural logarithm of the BO–Alb level was regressed upon the natural logarithm of exposure among the 30 exposed subjects, a strong effect of benzene exposure was observed ($R^2=0.612$; $p<0.0001$). Because the slope of the relationship between BO–Alb and benzene exposure was significantly less than one in log-space, we infer that production of benzene oxide was less than proportional to benzene exposure. Since benzene is a substrate for CYP2E1, these results are consistent with saturation of CYP450 metabolism. They indicate that deviations from linear metabolism began at or below benzene exposures of 10 ppm and that pronounced saturation was apparent at 40–50 ppm. To our knowledge, this is the first study to investigate the linearity of human metabolism of a carcinogen based upon protein adducts.

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1. Introduction

Benzene is a multi-site carcinogen in rodents that causes hematotoxicity and acute myeloid leukemia in humans [1]. Although the mechanism by which

benzene causes these effects is unknown, evidence strongly indicates that metabolism is required [2,3]. As summarized in Fig. 1, benzene is metabolized by CYP450 to benzene oxide (BO) [4,5], which exists in equilibrium with its valence tautomer oxepin. Subsequent enzymatic and non-enzymatic reactions of BO–oxepin give rise to other metabolites, notably, phenol, catechol, hydroquinone and the ring-opened muconaldehydes (ultimately transformed to *trans*, *trans*-muconic acid). Catechol and hydroquinone can

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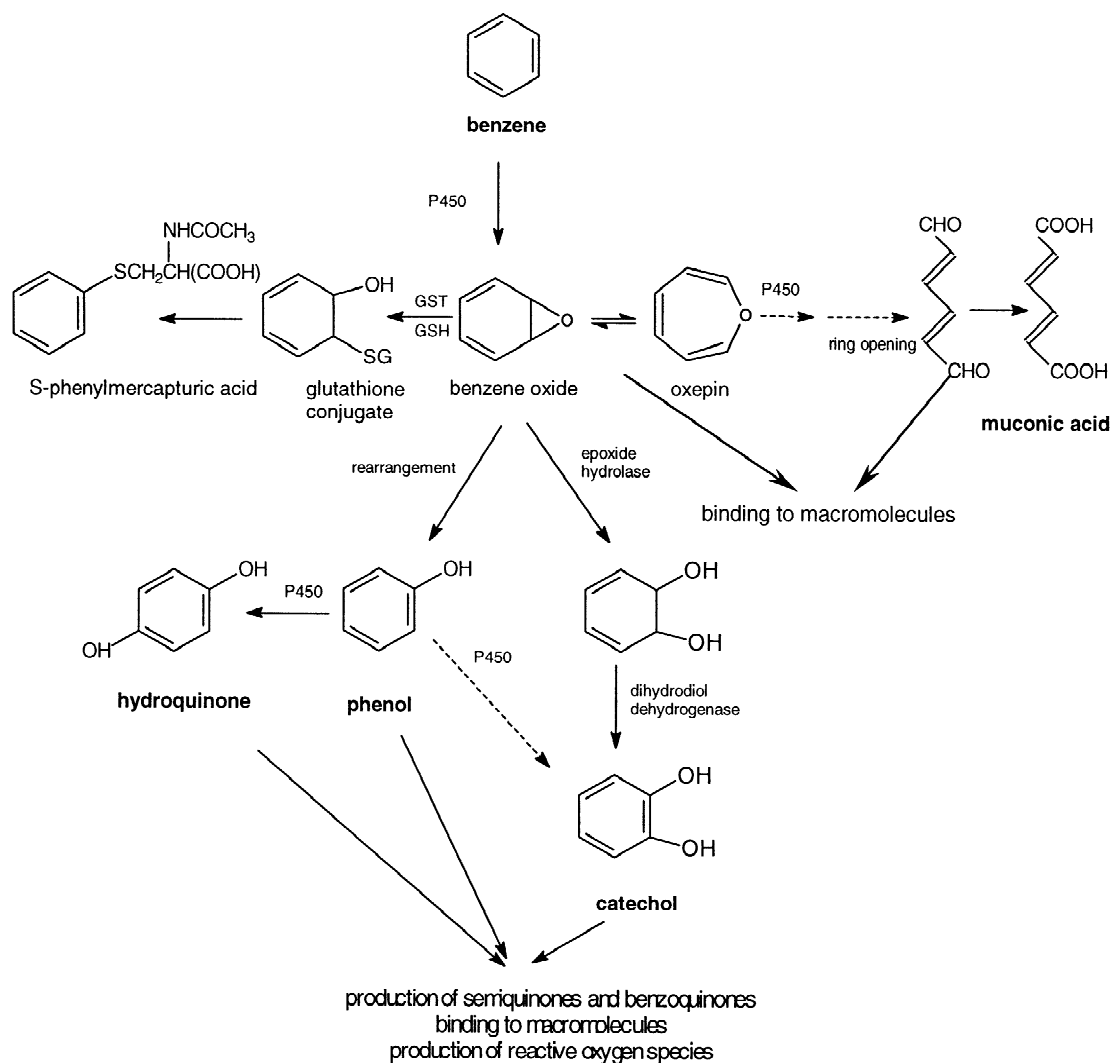


Fig. 1. Simplified scheme for metabolism of benzene in humans.

be oxidized to 1,2- and 1,4-benzoquinone, respectively, via the corresponding semiquinones.

The uncertain mechanism of benzene's toxicity arises from the possible roles of the many electrophilic metabolites (including BO, the muconaldehydes and the benzoquinones) as well as reactive oxygen species produced by redox cycling of catechol, hydroquinone and the benzoquinones [3]. Thus, even though there appears to be a causal link between human exposure to high levels of benzene (above 10 ppm) and leukemia, the prediction of human risks at lower levels of exposure is fraught

with uncertainty. Since airborne benzene is ubiquitous at low levels in the environment, typically at concentrations less than 10 ppb [6], and can reach thousand-fold higher levels in the workplace [7], the shape of the exposure–response relationship below 10 ppm represents a major quandary for environmental epidemiology.

Because the uncertainty surrounding benzene's exposure–response curve is unlikely to be resolved by conventional epidemiological studies, recent efforts have turned to biomarkers to shed light upon exposure–rate effects involving uptake, biotransfor-

mation and cell damage in humans exposed to benzene. Here the goal has been to elucidate relationships between benzene exposure and the corresponding levels of urinary metabolites, protein adducts, cytogenetic changes and blood abnormalities (e.g., see Refs. [8–11]).

Since the toxic effects of benzene are so clearly linked to metabolism, one promising avenue of inquiry involves the relationship between benzene exposure and levels of BO, from which all other metabolites arise (Fig. 1). However, direct measurement of BO in humans is impractical due to the reactivity of this compound in blood and by the limited sensitivity of assays [4]. Thus, recent efforts have employed adducts of BO with hemoglobin (Hb) and serum albumin (Alb) among benzene-exposed workers to make inferences about levels of BO in blood. (These adducts will henceforth be designated as BO–Hb and BO–Alb, respectively). As shown in Fig. 2, cysteinyl adducts are presumed to be formed from reactions of BO with free cysteinyl residues in proteins to give *S*-(2-hydroxycyclohexa-3,5-dien-1-yl) cysteine derivatives, which may persist or spontaneously dehydrate to *S*-phenylcysteine. Since BO–Alb and BO–Hb are stable in vivo [12], they accumulate over periods of 1 month (the mean residence time of human serum albumin, corresponding to a half life of 21 days) or 4 months (the life span of human erythrocytes), respectively. Hence, levels of these protein adducts reflect average concentrations of BO produced by exposure to benzene over 1 to 4 months [13]. Exposure-related increases in both BO–Hb and BO–Alb have been reported among workers exposed to benzene [11,14,15].

Because Alb adducts tend to be much more abundant than Hb adducts at a given exposure to benzene, BO–Alb is preferable for human biomonitoring [11,14]. We previously reported relationships between benzene exposures and levels of

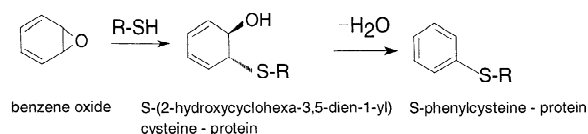


Fig. 2. Reaction of benzene oxide with a free cysteinyl residue of a protein (R-SH) to produce a protein adduct.

BO–Alb among 86 workers (43 currently exposed to benzene and 43 controls) in Shanghai China, in factories where benzene was used as a solvent [11,14]. Due to heteroscedasticity, we used weighted-least-squares regression to predict linear relationships between subject-specific benzene exposures (1.65–328 ppm) and the corresponding levels of BO–Alb. It was also necessary to adjust regression coefficients for respirator use by some workers. These factors, coupled with a high background level of adducts among controls (arising primarily from non-benzene sources), obscured the relationship between BO–Alb and benzene exposure. In the current study, we employ alternative statistical methods to investigate the linearity of the relationship between BO–Alb and benzene exposure among workers who did not wear respirators ($n=30$). The results indicate that the rate of production of BO–Alb begins to diminish at or below 10 ppm of benzene and approaches saturation at 40–50 ppm.

2. Experimental

The data used in this study were obtained from a previous investigation of protein adducts among 86 workers [11,14]. The methods are briefly summarized as follows.

2.1. Subjects

Forty-four benzene-exposed workers were recruited from three factories in Shanghai, China where benzene was used to solubilize natural rubber (factory 1), to manufacture adhesive tape (factory 2) and to paint wooden toys and boxes (factory 3); 44 control workers, who had been frequency matched with exposed subjects by gender and age, were recruited from a sewing machine manufacturing plant (factory 4) and an administrative facility (factory 5) in the same geographic region [8]. Since blood was not available for one of the exposed workers, complete sets of data were available for 87 of these frequency-matched subjects (43 exposed and 44 controls). Of these, 16 exposed subjects in factory 1 wore respirators and were excluded from analyses in the current study. Two additional workers were excluded from the present study based upon regres-

sion diagnostics, which revealed great influence upon coefficients and model fit. {One was a control worker for which laboratory notes indicated poor chromatographic resolution during gas chromatography–mass spectrometry (GC–MS) analysis and the other was a worker with very high exposure (328 ppm) who had unrealistically low levels of adducts as well as urinary benzene metabolites [11,14]}. In addition to these frequency-matched subjects, four additional workers with low exposures to benzene were included in the present study. Thus, data from 73 subjects were analyzed in the current investigation (30 exposed workers and 43 controls).

2.2. Exposure measurements

Passive monitors had been used to measure personal benzene exposures of each exposed subject during the full work shift on 5 consecutive days (two workers had six measurements) just prior to blood collection [8]. The geometric mean of these air measurements was used to estimate each subject's exposure to benzene; for the 30 exposed subjects the overall (geometric mean) exposure to benzene was 11.0 ppm benzene. Control subjects from factory 4 had been monitored for benzene exposure on a single day ($n=30$); only three of these measurements (0.047, 0.052 and 0.110 ppm) were above the limit of detection of 0.016 ppm. Control subjects in factory 5 had not been monitored and were assumed to have no exposure to benzene ($n=13$).

2.3. Adduct measurements

BO–Alb had been measured in a single sample of venous blood obtained from each subject, as described previously [11,14]. The assay, illustrated in Fig. 3, is based upon reaction of an *S*-phenylcysteine residue (in serum Alb) with trifluoroacetic anhydride

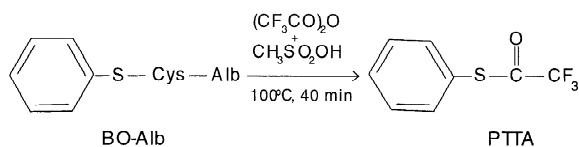


Fig. 3. Reaction of BO–Alb with trifluoroacetic anhydride and methanesulfonic acid to produce phenyltrifluoroacetate (PTTA).

and methanesulfonic acid to produce phenyltrifluoroacetate (PTTA), which was measured by GC–MS in the negative chemical ionization (NCI) mode [16,17]. [The initial adduct shown in Fig. 2, an *S*-(2-hydroxycyclohexa-3,5-dien-1-yl) cysteine derivative, dehydrated to form an *S*-phenylcysteine residue either spontaneously or under the acidic conditions of the assay]. Following isolation, 4–5 mg Alb were combined with 3–10 pmol [$^2\text{H}_5$]*S*-phenylcysteine (internal standard) and 200 μl of 100 mM ascorbic acid (to reduce adducts of the benzoquinones which were measured simultaneously as the corresponding hydroquinones). An 800- μl volume of trifluoroacetic anhydride and 20 μl of methanesulfonic acid were then added and the reaction mixture was heated to 100°C for 40 min. The remaining trifluoroacetic anhydride was removed under a stream of nitrogen. Following extraction with 1 ml hexane, the hexane layer was washed with 1 ml of 0.1 M Tris buffer (pH 7.5) and 2 \times 1 ml of deionized water. After concentrating the hexane layer to \sim 200 μl , 2–3 μl of each sample were analyzed by GC–MS in the NCI mode using selected ion monitoring of PTAA (m/z 206) and the derivatized internal standard [$^2\text{H}_5$]PTTA (m/z 211). A representative chromatogram is shown in Fig. 4 for Alb obtained from a worker exposed to 30 ppm benzene. The relative standard deviation of this assay had been estimated to be 36% based upon duplicate analyses of 48 specimens of Alb from these subjects [14]. Average levels of BO–Alb were assigned to those subjects with duplicate analyses.

2.4. Data analysis

Linear regression was performed using Proc REG of SAS system software (SAS Institute, Cary, NC, USA). To eliminate heteroscedasticity and to satisfy normality assumptions, regression employed (natural) logarithmic transformation of levels of BO–Alb and benzene exposure. Goodness of fit and influence were evaluated with standard residual diagnostics and normality plots. Regression analysis was performed with data only from exposed subjects ($n=30$). The regression was repeated after subtracting the GM level of 115 pmol/g Alb observed in controls ($n=26$ subjects with adjusted levels greater

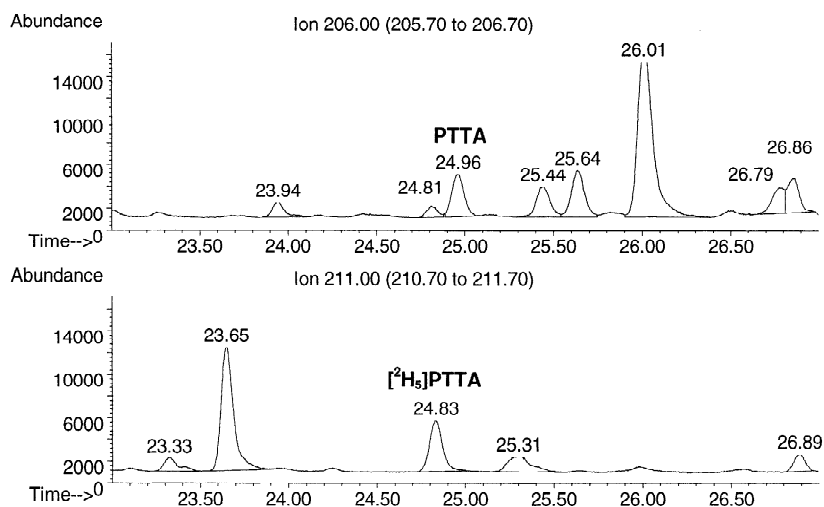


Fig. 4. GC–MS (negative-ion chemical ionization) selected-ion monitoring chromatogram of phenyltrifluoroacetate (PTTA) obtained by reaction of 4 mg of albumin, from a worker exposed to 30 ppm benzene, with trifluoroacetic anhydride and methanesulfonic acid. [$^2\text{H}_5$]S-Phenylcysteine (3 pmol) was added as the internal standard.

than zero). Controls were not included in the regression analysis because incomplete exposure data were available from unexposed subjects; i.e., while each exposed subject had five full-shift measurements some controls had only one measurement and others had no measurements. Within- and between-subject components of variance of benzene exposure were determined via one-way analysis of variance (ANOVA) of the logged exposure levels using Proc NESTED of SAS.

3. Results

Summary statistics, showing median values and ranges of benzene exposures and BO–Alb levels, are presented in Table 1. The level of adducts was much greater for exposed subjects [geometric mean (GM)=378 pmol/g protein, range: 52.7–2240 pmol/g Alb] than for controls (GM=115 pmol/g protein, range: 44.7–248 pmol/g Alb) ($p < 0.0001$, Wilcoxon rank sum test).

Levels of BO–Alb are depicted with individual exposures in Fig. 5A as a log–log plot. The figure shows that logarithmic transformation normalized the variance of adduct levels and that the fit of the straight-line model was reasonable. Simple linear

regression revealed a strong effect of benzene exposure according to the following relationship:

$$\text{BO-Alb (ln[BO-Alb, pmol/g protein])} = 4.89 + 0.434 * (\text{ln[benzene, ppm]})$$

($R^2 = 0.612$; $p < 0.0001$). After adjustment for background adducts the corresponding relationship was: $\text{Bo-Alb} = 4.38 + 0.497 * (\text{ln [benzene, ppm]})$ ($R^2 =$

Table 1

Benzene exposures and adduct levels among exposed and control subjects

Variable	Controls ($n = 43$)	Exposed ($n = 30$)
Exposure GM ^a	–	11.0
Exposure GSD ^a	–	5.76
Exposure range ^a	<0.016–0.110	0.160–302
BO–Alb GM ^b	115	378
BO–Alb GSD ^b	1.53	2.58
BO–Alb range ^b	44.7–248	52.7–2240

GM=Geometric mean; GSD=geometric standard deviation.

^a Benzene exposures (in ppm) based upon five daily measurements per exposed subject and 0–1 measurement per control subject; the GM and GSD shown for exposed subjects are based upon the mean values of individual workers logged exposures.

^b Benzene oxide–Alb adducts in pmol/g protein based upon one blood specimen per subject.

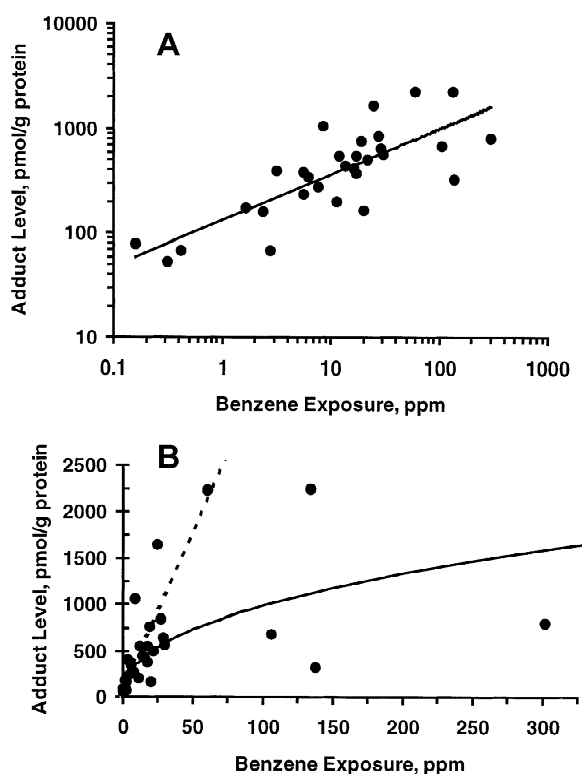


Fig. 5. Scatterplots of levels of BO–Alb versus benzene exposure for 30 subjects. (A) Log-space plot; the line represents the least-squares regression. (B) Natural-space plot; the solid curve represents the adduct level predicted from (A) and the dashed line represents a linear relationship between the adduct level and benzene exposure.

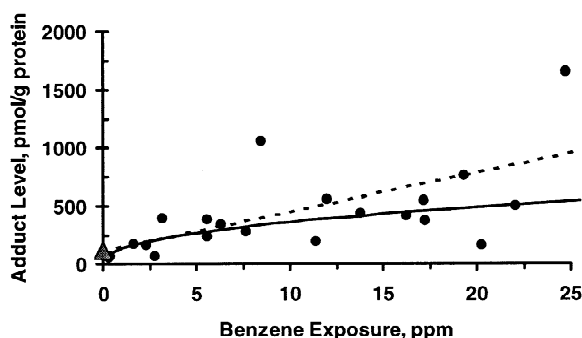


Fig. 6. Scatterplot of levels of BO–Alb versus benzene exposure for 30 subjects. (Curves are from Fig. 5B with axes modified to focus upon benzene exposures below 25 ppm). The solid curve represents the adduct level predicted from Fig. 5A; the dashed line represents a linear relationship between adduct level and benzene exposure; the triangle represents the geometric mean adduct level measured in control subjects.

0.359, $p=0.0012$, $n=26$). Since the estimated slope of 0.497 was less than one in log-space (95% C.I.: 0.217, 0.777), we infer that the rate of BO–Alb production diminished at higher benzene exposures (forming a plateau at high levels). The same data plotted in Fig. 5B show this plateau-shape for the corresponding relationship between adducts and benzene exposure in natural space. The dashed line in Fig. 5B represents the straight-line relationship that would be expected if adduct levels were strictly proportional to benzene exposure; here a slope of 39.5 pmol BO–Alb/g protein/ppm benzene was predicted. Fig. 6 presents the same relationship as Fig. 5B with the scale reduced to focus upon adduct production below 25 ppm of benzene; the GM adduct level among controls is designated by a triangle on the y-axis.

4. Discussion

This analysis indicates that levels of BO–Alb were less than proportional to benzene exposures over the range of 0.2–302 ppm. Since BO–Alb should reflect the average level of BO in the blood over a period of about 1 month prior to blood collection [13], this suggests that the rate of BO production diminished with increasing benzene exposure among these subjects. In fact, Fig. 6 indicates that the rate of adduct production began to decline noticeably in the range of 10 ppm benzene.

Since benzene is a substrate for human and rodent CYP2E1 [18–21], saturable metabolism would be expected at some level of exposure. However, given the paucity of human data, rates of benzene metabolism have been predicted from physiologically based pharmacokinetic models [22–24], which pointed to linear kinetics below 10 ppm [23] and to saturation above 100 ppm [22]. Our results, indicating nearly linear production of adducts below 10 ppm (Fig. 6) and substantial saturation at 40–50 ppm of benzene (Fig. 5B), are generally consistent with these pharmacokinetic models.

The estimated regression coefficient (i.e., 0.477 ($\ln[\text{pmol BO–Alb/g protein/ppm benzene}]$) for background-adjusted adduct levels assumes that benzene exposure was known without error. Since this was certainly not the case, the observed regression

coefficient was probably attenuated. However, assuming that errors in estimation of BO–Alb were uncorrelated with those of benzene exposure (which is reasonable given a 1-month residence time of BO–Alb and daily measurement of exposure), it is possible to adjust the regression coefficient based upon the variance components of benzene exposure within and between subjects [25]. From one-way ANOVA of the (logged) exposure data, the estimated ratio of within- to between-person variance components was 0.332 for the 30 subjects in our sample (with five measurements each). Based upon Ref. [25], we predict an expected value for the “true” regression coefficient of $0.477 \cdot (1 + [0.332/5]) = 0.509 \cdot (\ln[\text{pmol BO–Alb/g protein/ppm benzene}])$, an attenuation of only 6.7%. Thus, if the individual air measurements were representative of exposures over the month preceding blood collection (the residence time of BO–Alb), the straight-line relationship shown in Fig. 5A should be reasonable. By extension, the non-linear relationships shown in Figs. 5B and 6 should be relatively unbiased over the observed ranges of benzene exposure.

Production of total urinary metabolites was also observed to be non-linear with benzene exposure among these same workers [9,26]. However, in order to estimate total urinary metabolites it was necessary to add the levels of all major urinary products of benzene, each of which had been measured independently with error. Thus, the variability of total urinary metabolites was probably greater than that of BO–Alb. Also, because the residence times of urinary metabolites are less than 1 day, the errors in these estimated (urinary) levels would be correlated with those of benzene exposure from day to day. In this case, the effect of measurement error on the estimated regression coefficient would be more profound than that for stable protein adducts. For these reasons, we regard the inferences derived from BO–Alb, about the overall rate of benzene metabolism, to be more accurate and precise than those from urinary data. This inherent strength of protein adducts for elucidating rates of human metabolism has not, to our knowledge, been exploited heretofore.

Finally, we recognize that our finding about the non-linearity of human benzene metabolism is based upon only 30 subjects from a single investigation. We are currently conducting a similar study among a

larger population of workers, to more thoroughly explore human metabolism of benzene over the critical exposure range of 1–10 ppm.

Acknowledgements

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