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Tissue distribution and macromolecular binding of extremely low doses of [^{14}C]-benzene in $B_6C_3F_1$ mice

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The tissue distribution and macromolecular binding of benzene was studied over a dose range spanning nine-orders of magnitude to determine the nature of the dose–response and to establish benzene's internal dosimetry at doses encompassing human environmental exposures. [^{14}C]-Benzene was administered to $B_6C_3F_1$ male mice at doses ranging between 700 pg/kg and 500 mg/kg body wt. Tissues, DNA and protein were analyzed for [^{14}C]-benzene content between 0 and 48 h post-exposure (625 Ng/kg and 5 μg/kg dose) by accelerator mass spectrometry (AMS). [^{14}C]-Benzene levels were highest in the liver and peaked within 0.5 h of exposure. Liver DNA adduct levels peaked at 0.5 h, in contrast to bone marrow DNA adduct levels, which peaked at 12–24 h. Dose–response assessments at 1 h showed that adducts and tissue available doses increased linearly with administered dose up to doses of 16 mg/kg body wt. Tissue available doses and liver protein adducts plateau above the 16 mg/kg dose. Furthermore, a larger percentage of the available dose in bone marrow bound to DNA relative to liver. Protein adduct levels were 9- to 43-fold greater than DNA adduct levels. These data show that benzene is bioavailable at human-relevant doses and that DNA and protein adduct formation is linear with dose over a dose range spanning eight orders of magnitude. Finally, these data show that the dose of bioactive metabolites is greater to the bone marrow than the liver and suggests that protein adducts may contribute to benzene's hematoxicity.

Introduction

Benzene has been one of the most heavily utilized industrial solvents in the last 100 years. It is also present in cigarette smoke and unleaded gasoline, is a product of combustion, and is present in rural and urban air (1). In animals, benzene has been shown to be a multi-potential carcinogen, causing cancers in multiple species, at multiple sites and in both sexes (1–4). In humans, occupational exposure to benzene is associated with the induction of acute myelogenous leukemia and may also be associated with other types of cancer such as lung, chronic myeloid or lymphocytic leukemia, and lymphocyte-derived malignancies (4). However, these data come from high dose animal and human occupational exposures. While it is clear that high dose benzene exposure is a threat to human health, it is not clear how to extrapolate these data to humans exposed to low levels of benzene via their environment.

Since tumor studies are only carried out at high doses, endpoints other than tumor formation are routinely used to aid in extrapolating to lower doses for estimating human cancer risk. Two such endpoints, protein adducts and DNA adducts, are recognized as biomarkers of exposure and are thought to be valid measures of the active dose to target molecules (5). DNA adducts have been reported to correlate with mutagenic activity and tumorigenicity (6,7). Covalent binding of xenobiotics to proteins can serve as surrogates for DNA adducts and disrupt functionality, which may also contribute to neoplastic transformation (8).

While the exact mechanism for benzene-induced hematoxicity is still unknown, it is thought to involve metabolism of benzene to reactive intermediates and adduct formation (4,9,10,11). Metabolic profiles for benzene vary significantly with dose, with extremely high doses favoring the formation of putatively less toxic metabolites (12). Such dose-related alterations in the metabolite profile can affect the apparent tissue distribution and clearance kinetics of the compound or its metabolites. Thus, an important question is how the administered dose of benzene and the tissue distribution of benzene relate to the formation of macromolecular adducts within these tissues, particularly at doses relevant for human environmental exposures.

Until recently, the measurement of adducts at human-relevant doses of environmental contaminants has been difficult, particularly with benzene. High doses or large sample sizes are typically needed to achieve adduct levels measurable by traditional techniques. We recently reported on the use of accelerator mass spectrometry (AMS*) to measure DNA adducts following administration of low doses of carcinogens (13,14). AMS is a nuclear physics technique for measuring isotope ratios with high sensitivity, selectivity and precision (15). AMS sensitivity with ^{14}C-labeled carcinogens is in the low attomole (10^{-18} mol) range, with DNA adduct detection sensitivity (depending on specific activity) of a few adducts/10^{12} nucleotides (14,16). In this study, we used AMS to quantify the level of benzene DNA and protein binding in the liver and bone marrow of rodents given [^{14}C]-benzene at doses as low as 700 pg/kg body wt. We also measured the effect of administered dose on the tissue distribution of the benzene dose. The purpose of this work was to: (i) determine if benzene adducts are present in macromolecular DNA and protein at low doses, (ii) determine how adduct levels relate between liver, where significant amounts of benzene metabolism occurs, and bone marrow, which is the primary target organ for cancer, and (iii) to assess the linearity of the dose–response from the high doses where rodent tumor studies have been conducted to the low doses routinely encountered in the environment by the human population.

***Abbreviations:** AMS, accelerator mass spectrometry; MOPS, 3-[*N*- morpholino]propanesulfonic acid; DTT, dithiothreitol; PCA, perchloric acid; PBS, phosphate buffered saline; HPLC, high-performance liquid chromatography; AUC, areas under the curve.

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Materials and methods

Chemicals

[U-^{14}C]Benzene (50.3 mCi/mmol), obtained from Sigma Chemical Co. (St Louis, MO), was checked for radiopurity using a Hewlett Packard model 1084B, high-performance liquid chromatography (HPLC) system equipped with a Rainin (Emeryville, CA) C-18 column (10 mm internal diameter ×25 cm). The analysis was carried out isocratically (70% methanol/30% water) at a flow rate of 1 ml/min. Detection was carried out by UV absorption at 254 nm and liquid scintillation counting of HPLC fractions collected at 1 min intervals. Under these conditions, benzene eluted at ~7 min. Radioactivity associated with the benzene peak was >99%. RNase A (E.C. 3.1.27.5), RNase T1 (E.C. 3.1.27.3), proteinase K (E.C. 3.4.21.14), 3-[N-morpholine] propanesulfonic acid (MOPS), urea and Triton-X 100 were also obtained from Sigma Chemical Co. Benzene (used to dilute the specific activity of [^{14}C]-benzene) and tributyrin were obtained from Aldrich Chemical Co. (Milwaukee, WI). Protein assay reagent and dithiothreitol (DTT) were obtained from Pierce (Rockford, IL). All other chemicals were analytical grade.

Animals

$B_6C_3F_1$ male mice (~8 weeks of age; 25–30 g) were obtained from Simonsen Laboratories (Gilroy, CA) and acclimatized for a minimum of 1 week in an AAALAC-accredited animal care facility before use. Animals were housed three per cage in filter top, polycarbonate cages with hardwood chip bedding. They were given water and food *ad libitum*, kept on a 12 h light/dark cycle, and maintained at ~22°C throughout the study.

Preparation of dosing stocks and treatment of mice

Dosing solutions were prepared by serial dilution of [^{14}C]-benzene with filter-sterilized corn oil. For benzene doses above 500 ng/kg, the specific activity was adjusted using unlabeled benzene. The final specific activities for each dose level were as follows: 500 mg/kg at 5.1×10^{-5} mCi/mmol, 15.78 mg/kg at 0.00158 mCi/mmol, 500 μg/kg at 0.051 mCi/mmol, 15.78 μg/kg at 1.52 mCi/mmol, 500 ng/kg and below at 50.3 mCi/mmol. Each dosing stock concentration was verified by liquid scintillation counting prior to administration.

For animal treatments, [^{14}C]-benzene was administered to the animals by i.p. injection (0.2 ml/25 g body wt). The maximum amount of radioactivity administered to each animal was 8–10 nCi. Mice treated with 500 pg/kg and 15.78 ng/kg benzene received 8 pCi and 254 pCi, respectively. Mice treated with 625 Ng/kg and 5 μg/kg were euthanized at 0.5, 1, 3, 6, 24 and 48 h post-administration by CO_2 asphyxiation followed by cardiac puncture to collect blood. Mice utilized in dose-response studies were euthanized at 1 h. Tissues were removed using disposable forceps and scalpels and blotted free of blood as described by Frantz *et al.* (17). Tissues were not perfused to remove residual blood and blood volume corrections were not performed. Bone marrow was collected by flushing each femur with ~1 ml of phosphate buffered saline (PBS), pH 7.4. All tissues were frozen at –20°C until processed.

DNA isolation

DNA was isolated from liver and bone marrow as described by Frantz *et al.* (17). Briefly, ~100 mg of pulverized liver or bone marrow from 1–2 femurs was solubilized overnight in 4–5 ml of freshly made lysis buffer containing 4 M urea, 10 mM Tris–HCl (pH 8.0), 1% Triton-X 100, 10 mM DTT, 100 mM NaCl and 800 μg/ml proteinase K in a 37°C shaking water bath. Samples were centrifuged to remove undigested tissue, and the supernatants were forced through a 26-g, 1/2-inch (1.3 cm) needle 10 or more times to shear the DNA. RNase A (0.5 mg/ml, final concentration) and T1 (2500 units, final concentration) were then added and incubated for an additional 30–60 min at 37°C to digest RNA. Aliquots of 1 M MOPS (50 μl/ml) and 5 M NaCl (180 μl/ml) were then added to the lysates and the samples were loaded onto Qiagen tip 100 anion exchange columns (Qiagen Inc., Chatsworth, CA) that had been pre-equilibrated with a loading buffer (750 mM NaCl, 50 mM MOPS, 15% EtOH and 0.15% Triton-X 100, pH 7.0). After the lysate was loaded, the column was washed with three column-volumes washing buffer (1 M NaCl, 50 mM MOPS, 15% EtOH, pH 7.0) and the DNA was eluted in one column-volume of elution buffer (125 mM NaCl, 50 mM MOPS and 15% EtOH, pH 8.0). One column-volume of ice-cold isopropanol was added to the elution buffer containing the DNA and the samples were stored at −20°C for 12 h. At the end of this 12-h storage the DNA was pelleted by centrifugation at 3000 r.p.m. for 4–5 h at –4°C. The DNA pellet was washed with 70% EtOH and reconstituted in ultrapure water (18 MOhm). This procedure yielded ~100 μg of DNA/100 mg tissue with an A260/A280 ratio of 1.7–1.9. The DNA concentration was determined by UV absorption at 260 nm, assuming an A260 of 1.0 = 50 μg/ml DNA.

Acid precipitation of macromolecules

Approximately 100 mg of liver or bone marrow from two femurs was pulverized and lysed using a procedure similar to that used for DNA isolation, except that no proteinase K was added. After centrifugation, insoluble tissue was removed and 70% perchloric acid (PCA) in water was added to the supernatant to give a final concentration of 5% PCA. The samples were centrifuged at 3000 r.p.m. for 15 min at room temperature, and the pellet was resuspended in 1–2 ml 5% PCA. The precipitate was then pelleted by centrifugation and washed twice in 50% methanol and EtOH/ether (1:1 v/v). The resultant pellet was allowed to air dry before resuspension in ultrapure (18 MOhm) water for measurement by AMS. PCA precipitate from bone marrow was solubilized in 1 N KOH by overnight incubation at 37°C followed by adjustment to pH 7.0 with HCl. The protein content of the bone marrow was then determined with the Bradford microassay (Pierce) by following the manufacturer's instructions and using bovine serum albumin as a standard. Macromolecules isolated using this procedure are hereafter referred to as protein, although this method does not provide a pure protein preparation.

Preparation of samples for AMS measurement

Preparation of samples for radiocarbon analysis by AMS requires conversion of the samples to graphite. All tissues, DNA isolates, protein fractions and reagents were handled carefully to avoid radiocarbon cross-contamination. This required the use of disposable materials for any item that might come into contact with a sample (e.g. gloves, tubes, bottles containing buffers, bench paper). For frozen tissues, samples were cut into ~25-mg pieces by using disposable needles or scalpels and placed into 6×50 mm quartz tubes. Bone marrow in PBS (1 ml), DNA in water, protein, and whole blood (50 μl) were pipetted into 6×50 mm quartz tubes using aerosol-resistant pipette tips. For small samples, such as the DNA isolates or the protein isolated from bone marrow, 1–2 mg of carbon in the form of tributyrin (diluted in methanol) was added to bring the total carbon content to 1 mg (an amount known to produce uniform graphite). All samples were then dried either under a stream of N_2 gas or by vacuum centrifugation.

The dried samples were converted to graphite by a two-step process described by Vogel (18). Briefly, the dried samples were oxidized to CO_2 by heating at 900°C for 4 h in the presence of copper oxide. The CO_2 was then cryogenically transferred to a second quartz tube under vacuum using a Nalgene 'Y' connector and reduced to filamentous graphite in the presence of cobalt, titanium hydride and zinc powder. The resultant graphite was placed in the ion source of the spectrometer and analyzed using previously published protocols (16).

AMS analysis

AMS measures the ratio of a rare isotope relative to a stable isotope of the same element. For [^{14}C]-benzene analysis, we measured ^{14}C relative to ^{13}C and normalized to the $^{14}C/^{12}C$ ratio of 1950 carbon using the Australian National University sugar reference standard (16). The ratios are converted to picograms of [^{14}C]-benzene/g tissue, after subtraction of the background radiocarbon contributed from the sample and carrier, and correction for the specific activity of the benzene and the carbon content of the original sample. Carbon contents of the samples were determined using a C:N:S analyzer (Carlo-Erba NA1500, series 2). Tissues, including blood and bone marrow, were determined to have carbon content ranging between 11% and 17% (wet weight). DNA and protein were determined to contain 29% and 48% carbon (dry weight) respectively.

In these studies, we analyzed radiocarbon content in tissues, DNA and acid precipitable macromolecules. Since AMS only measures the radiocarbon label in the fraction analyzed, we cannot distinguish between the parent benzene moiety or its metabolites, nor can we distinguish between covalent and non-covalent interactions. Thus, data are collectively referred to as pg benzene equivalents/g of tissue, DNA or protein.

Statistical analysis

Three animals were used at each time point and at each dose level. Regressions of the natural logarithm transformation of benzene levels in the tissue and macromolecules against the natural logarithm transformation of dose were calculated using the JMP statistical package (SAS Institute,Cary, NC). Hypothesis testing for linearity and for equality of slopes used a two-sided *P*-value equal to 0.05. In order to stabilize the variance, and because the data were measured by subtracting a baseline concentration of radiocarbon, we added a constant of 0.1 pg benzene/g sample to the measured benzene concentrations before taking the logarithms. All samples were analyzed multiple times by AMS (n = 3 in most cases) and averages of each were used for statistical analysis. We also omitted the highest dose level from the analysis (except where noted in Table II) because of its clear non-linear relationship. For the dose–response analysis, the assumption of independence is not met because the same set of animals provided all tissues (except for bone marrow tissue and bone marrow protein, which were measured in a separate set of animals). Therefore, equality of slopes was tested by fitting the data to a multivariate regression model.

Table I. Kinetics of [^{14}C]-benzene uptake and clearance in various tissues following i.p. administration (pg [^{14}C]-benzene equivalents/g tissue)[a]

Time (h)	Liver	Kidney	Blood	Bone marrow
0	124.1 ± 145.5	50.2 ± 45.5	42.6 ± 33.4	
0.5	392.4 ± 102.1	564.7 ± 280.4	149.6 ± 71.1	100.1 ± 10.6
1	980.7 ± 193.4	490.2 ± 152.4	91.5 ± 22.8	59.9 ± 22.7
3	419.2 ± 100.9	437.0 ± 112.8	68.8 ± 50.5	24.1 ± 5.5
6	132.2 ± 37.5	135.0 ± 27.2	14.6 ± 2.4	17.2 ± 5.2
12	59.4 ± 35.1	85.7 ± 20.7	5.9 ± 2.7	9.2 ± 3.9
24	83.7 ± 13.7	68.4 ± 25.1	7.0 ± 1.5	7.0 ± 2.0

Time (h)	Lung	Spleen	Thymus	Heart
0	29.9 ± 27.0	21.4 ± 20.2	8.0 ± 6.7	12.1 ± 9.8
0.5	123.9 ± 53.5	61.7 ± 29.3	55.2 ± 29.3	58.9 ± 27.9
1	79.9 ± 23.6	82.9 ± 54.0	36.5 ± 10.9	35.4 ± 4.6
3	56.1 ± 29.1	34.4 ± 4.3	27.3 ± 6.9	26.5 ± 6.0
6	29.7 ± 17.0	15.7 ± 2.7	11.8 ± 1.3	10.4 ± 1.4
12	10.7 ± 2.8	6.6 ± 2.8	5.6 ± 0.9	4.0 ± 1.5
24	9.5 ± 1.4	7.1 ± 0.8	5.1 ± 0.4	4.5 ± 0.4

[a]Data represent the mean of three animals/time point ± SD with values for unexposed controls subtracted. The radiocarbon levels in unexposed animals were consistent with the $^{14}C/^{12}C$ ratio of present-day carbon ($^{14}C/^{12}C = 1.14\times10^{-12}$).

Results

Tissue distribution and kinetics

Table I shows the tissue distribution of [^{14}C]-benzene equivalents as a function of time for mice given a benzene dose of 628 ng/kg body wt i.p. The highest levels of ^{14}C-benzene equivalents were found in the liver and kidney, followed by the lung, blood and bone marrow. [^{14}C]-Benzene equivalents in the liver reached a peak concentration of almost 1000 pg [^{14}C]-benzene equivalents/g tissue 1 h post-exposure. The peak concentrations of [^{14}C]-benzene equivalents in the kidney were 1.7-fold lower than in the liver. The lowest benzene levels were found in the spleen, thymus and heart. Peak radiocarbon levels in all tissues were observed at 0.5 or 1 h post-exposure, although some tissues may have reached maximum levels before the 0.5 h time point or between the 1 h and 3 h time points.

The clearance of benzene and its metabolites from the tissues was rapid. A small fraction (5–12%) of the maximum tissue concentration remained in the tissues 24 h post-exposure (Table I). At 24 h after exposure, the concentrations of [^{14}C]-benzene equivalents were similar in all tissues except the liver and kidney, where benzene levels were still 10-fold greater than in the other tissues.

Figure 1 illustrates the amount of [^{14}C]-benzene equivalents associated with liver DNA at time points between 0 and 48 h post-exposure following administration of benzene doses of 628 ng/kg and 5 μg/kg body wt. Within 30 min of dosing, liver DNA binding reached a maximum of 166.5 ± 106.0 pg/g at the higher dose. At the lower dose (628 ng/kg), liver DNA binding levels were proportionately less (16.9 ng/kg) at its peak. At both the doses, the adduct levels declined steadily after 30 min post-exposure. At the low and high doses respectively, the levels were 4.3 ± 2.0 and 46.9 ± 6.3 pg [^{14}C]-benzene equivalents/g DNA 12 h post-exposure. Radiocarbon levels in the DNA remained essentially unchanged at subsequent time points to 48 h.

A very different response was obtained in the bone marrow DNA (Figure 1). The radiocarbon content of bone marrow DNA was low between the 0 and 3 h time points.

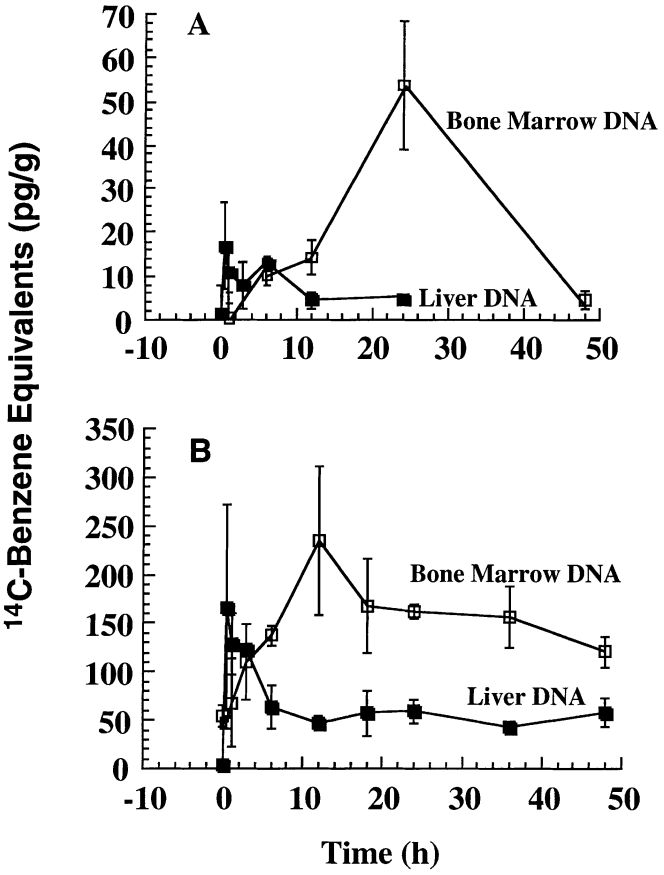

Fig. 1. Uptake and distribution of [^{14}C]-benzene equivalents in male $B_6C_3F_1$ mice by liver DNA and bone marrow DNA. Dosing was by i.p. administration of 628 ng (**A**) and 5 μg/kg (**B**) [^{14}C]-benzene in corn oil. Data represent mean ± SD of three animals/time point.

The variation in the data is high at these early time points because of the low radiocarbon content in the samples. However, radiocarbon levels in the bone marrow DNA steadily increased between 3 and 12 h, peaking at levels of

234 ± 76.4 pg [^{14}C]-benzene equivalents/g DNA (5 μg/kg dose level) 12 h post-exposure and at 53.8 ± 14.9 pg/g (628 ng/kg dose level) 24 h post-exposure. These levels are higher than the peak adduct levels measured 1 h post-exposure in the liver DNA.

Since binding kinetics were clearly different for liver and bone marrow, we calculated the areas under the curve (AUC) to estimate the total bioactive dose to each tissue. AUCs for DNA binding using the data presented in Figure 1 were calculated using the trapezoidal rule (19) from zero to time 't' (24 h for the liver and 48 h for the bone marrow) without extrapolation according to the following calculation:

$$AUC = \Sigma[(C_1 + C_2)/2\times(t_2 - t_1) + (C_2 + C_3)/2\times(t_3 - t_2) + ... (C_{x-1} + C_x)/2\times(t_x - t_{x-1})]$$

The AUCs calculated using this algorithm are proportional to dose in the liver. The AUC to dose ratio in liver is 0.3 at both the high and low doses. In the bone marrow, however, the AUC:dose ratio is 27% higher at the low dose.

Dose–response relationships

Table II gives the dose–response data for the [^{14}C]-benzene equivalents in tissues over a dose range spanning nine orders of magnitude. A linear relationship ($P < 0.05$) exists between administered dose and the level of benzene equivalents in the tissues at doses below 16 mg/kg. Furthermore, the slopes of the linear fits are parallel ($P < 0.05$). Saturation of absorption or metabolism was evident by the deviation from linearity in all tissues at the highest dose (Table II).

The dose–response relationships of benzene equivalents bound to liver DNA, bone marrow DNA and acid precipitable macromolecules (protein) are shown in Figure 2. DNA and protein were isolated 1 h after exposure for both the liver and bone marrow in order to make direct comparisons of the data among tissues. This time point was chosen to maximize the probability of measured adducts in both tissues. No saturation is evident over the entire dose range studied (500 mg/kg to 700 pg/kg) in either liver or bone marrow DNA. Radiocarbon levels in the DNA at the 500 mg/kg dose reached 11.5 and 6.8×10^6 pg ^{14}C-benzene equivalents/g DNA in the liver and bone marrow, respectively. At the dose of 500 ng/kg ^{14}C-benzene, these levels decreased to 17.20 pg [^{14}C]benzene equivalents/g DNA in the liver and 3.6 pg ^{14}C-benzene equivalents/g DNA in the bone marrow. Radiocarbon levels at doses below 500 ng/kg could not be distinguished from the background.

Figure 2B shows the protein adduct dose–response relationship. A linear relationship was found in both liver and bone marrow for benzene doses ranging from 700 pg/kg to 16 mg/kg body wt. At the high dose (500 mg/kg benzene), liver protein adducts exhibited saturation similar to that seen in the whole tissue. Protein in the bone marrow, however, did not exhibit saturation. Deviation from linearity in the bone marrow protein binding kinetics is seen at the lowest dose. This result is likely due to contamination, given the large uncertainty in the measurements, and has been omitted from further consideration in this study.

Table II. Dose–response relationship of [^{14}C]-benzene equivalents in male $B_6C_3F_1$ mice 1 h after i.p. administration

[^{14}C]-Benzene equivalents, pg/g tissue[a,b,c,d] Dose (ng/kg)	Liver	Kidney	Blood	Bone marrow[e]
500064020.5	59820347.1 ± 5592447.0	46302189.2 ± 9563868.3	22859794.7 ± 5051111.3	2520159.1 ± 566265.1
15782020.5	15836406.7 ± 3206380.4	18467986.6 ± 13298040.2	3510364.0 ± 860217.5	n/a
499263.9	458372.1 ± 251031.3	435342.6 ± 126233.0	138810.9 ± 87890.0	44196.8 ± 16418.9
15782.0	18746.9 ± 2487.2	14526.3 ± 1360.9	5325.4 ± 2828.2	n/a
540.3	543.5 ± 92.5	512.4 ± 191.4	139.6 ± 58.3	36.0 ± 9.4
18.3	27.2 ± 5.6	32.1 ± 21.9	15.7 ± 14.5	0.9 ± 0.2
0.7	0.4 ± 0.2	0.4 ± 0.0	0.0 ± 0.0	n/a

[^{14}C]-Benzene equivalents, pg/g tissue Dose (ng/kg)	Lung	Spleen	Thymus	Heart
500064020.5	19055766.7 ± 899845.2	8394521.9 ± 958579.2	5399735.6 ± 599461.2	6835393.1 ± 1122843.2
15782020.5	2320340.1 ± 326711.1	1414067.0 ± 246721.6	1030709.1 ± 288312.9	984392.4 ± 95114.1
499263.9	89604.9 ± 18701.3	47818.7 ± 15152.0	47225.2 ± 7167.8	45547.8 ± 20206.8
15782.0	3117.8 ± 510.6	1892.3 ± 231.6	1164.3 ± 37.6	1480.4 ± 162.4
540.3	90.1 ± 26.3	62.1 ± 25.0	21.3 ± 12.3	35.7 ± 5.8
18.3	5.5 ± 3.3	2.4 ± 0.5	2.0 ± 0.4	2.7 ± 2.3
0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a]Data represent the mean of three animals ± SD, with the exception of thymus (628 ng/kg, $n = 2$). Values for 18.3 ng/kg dose represent data from two independent experiments ($n = 8$ and $n = 9$). Values for 0.7 ng/kg dose represent four animals/dose. For the data shown the background contribution of radiocarbon (i.e. the amount of radiocarbon in undosed animals) has been subtracted.

[b]Regression parameters were fit to the model $y = a(x)^b$, where a = intercept and b = slope. Parameters were generated using all data except for the omission of the highest data point, where saturation was evident. Individual parameters for each tissue are as follows (intercept, slope): liver (1.155, 0.9895), kidney (1.207, 0.9825), blood (0.467, 0.9553), bone marrow (0.044, 1.0515), lung (0.271, 0.9630), spleen (0.171, 0.9553), thymus (0.140, 0.9460) and heart (0.101, 0.9795).

[c]Statistical tests of whether the regression slopes were equal to 1 were not rejected except for bone marrow, which had a slope significantly >1 and lung, spleen and thymus, which had slopes significantly <1.

[d]Regression goodness-of-fit tests showed significant non-linearity for liver, blood and thymus, but the practical size of this effect is quite small, as evidenced by plots of the data.

[e]Values for bone marrow tissue were obtained from independently replicated sets of experiments. All other measurements were made from the same animals.

Discussion

In this study we have explored the dosimetry of benzene over a dose range spanning likely human environmental exposures (20) and rodent bioassay doses. We find that benzene, when administered i.p. is rapidly absorbed into the general circulation and binds to both DNA and protein even at doses approximating human environmental exposure levels. We also find that the total tissue available benzene doses scale with a linear function of administered dose between doses ranging from ~700 pg/kg to 20 mg/kg body wt. The bioactive component of that dose in bone marrow and liver, using adducts as surrogates, also scale as a linear function of dose below the 20 mg/kg body wt dose.

The dose–response data, which was collected at a single time point, suggests a slightly greater level of adducts in liver relative to bone marrow. However, time integrated adduct levels clearly show that the total bioactive dose in these tissues is actually greater in the bone marrow. This measure of bioactive dose is also dose-dependent within the human exposure range. Additionally, the peak adduct levels attained in the bone marrow (12–24 h post exposure) were ~1.5–3 times greater than peak adduct levels attained in the liver (1 h post-exposure). This relationship shows that kinetic differences exist between these tissues and this would not have been evident if we relied only on the measurement of adduct levels at a single time point after administration of the dose. Thus, time integrated adduct levels correlate with the primary target site for tumors and are a more representative measure of the potential for benzene damage in the tissues than a single time point measurement.

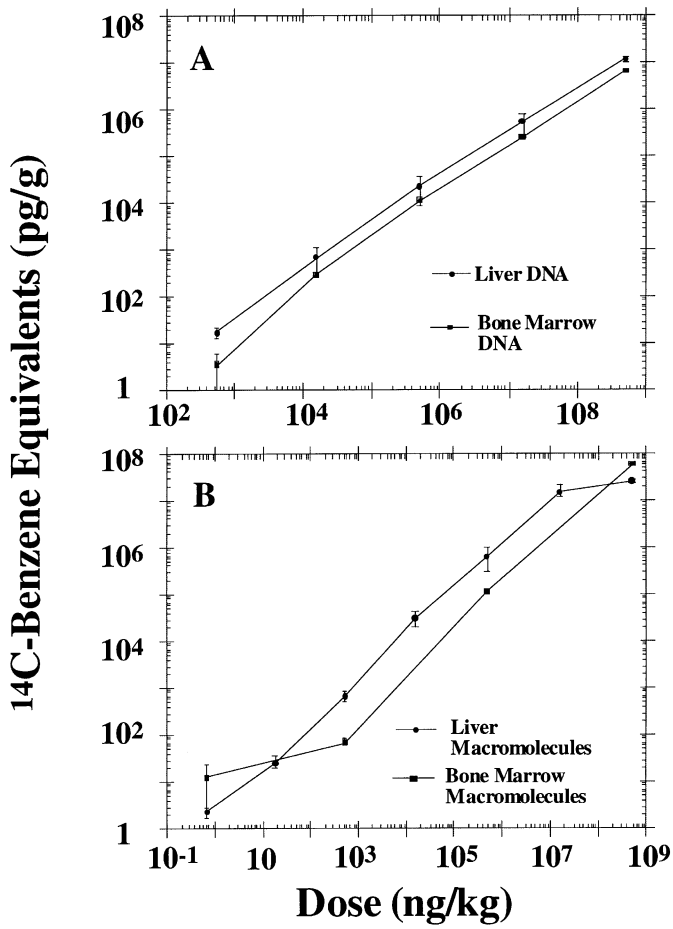

Fig. 2. Dose–response relationship of [^{14}C]-benzene equivalents in liver and bone marrow DNA (**A**) and macromolecules (**B**) from male $B_6C_3F_1$ mice 1 h after i.p. administration of various doses of [^{14}C]-benzene. Values represent mean ± SD of three animals/time point, with the exception of liver DNA (500 mg/kg dose, $n = 2$) and bone marrow DNA (15.78 μg/kg dose, $n = 2$). Bone marrow macromolecules showed significant non-linearity at the lowest dose, but the most likely explanation for this is inadvertent carbon contamination. Statistical tests for equality of slopes show that the slope for bone marrow DNA is marginally significantly different from liver DNA when a multivariate regression approach is used ($P < 0.05$).

These data imply that either bioactive benzene metabolites or DNA adducts accumulate in the bone marrow with time. This implication is consistent with other reports showing that benzene metabolites such as hydroquinone and catechol concentrate in the bone marrow (21). An additional hypothesis that would explain this result is the *de novo* metabolism of reactive benzene intermediates directly in the bone marrow from benzene or its intermediates brought to the bone marrow via the general circulation. Our data do not allow us to differentiate between these two possibilities but work is in process to measure the toxicokinetics of the individual benzene metabolites in these tissues at low dose. Regardless of the mechanism, while the distribution of total benzene metabolites to the tissues does not appear to be indicative of target organ toxicity, a very different picture is obtained when peak benzene adduct levels or the time integrated adduct concentration among tissues are compared.

Our data also raise questions about the stability of the DNA adducts. Benzene DNA adduct levels in the livers of animals given 628 ng/kg and 5 μg/kg benzene peaked within an hour of exposure, remained relatively unchanged for 6 h, and then declined. This data suggests that a fraction of the DNA adducts are unstable. The significance of this observation is uncertain, since the benzene DNA adduct levels in liver at these doses were extremely low and had a large variance between animals. The observation that bone marrow DNA adducts, which were significantly greater than the liver adducts, also appear to decline after 12–24 h support the suggestion of DNA adduct instability.

Overall, the binding of benzene metabolites to DNA is weak relative to other carcinogenic agents. Our data are consistent with those from other direct labeling techniques: benzene produces a covalent binding index of ~10 (7,10,22), a value typical for weak carcinogens. For comparison, the covalent binding index of aflatoxin, a strong DNA binding agent and carcinogen, is reported as ~17 000 (7). Studies using ^{32}P-postlabeling have, for the most part, failed to detect aromatic benzene adducts, even following prolonged dosing regimens (22,23) where blood dyscrasias or cell toxicity are evident. The most likely explanation is that benzene is forming tightly associated but non-covalently bound adducts, non-aromatic adducts or adducts with the sugar-phosphate backbone (22). Hydroquinone (a benzene metabolite) has recently been shown to form N^2-(4-hydroxyphenyl)-2′-deoxyguanosine-3′-phosphate hydroquinone *in vitro* with DNA ^{32}P-postlabeling studies suggest that this adduct may form *in vivo* as well (23,24). To our knowledge no other biologically relevant adducts for benzene have been characterized, so the reasons for the difficulty in detecting benzene adducts cannot be explained.

These data also show that benzene binds to proteins to a much greater extent than to DNA. In view of the knowledge that benzene is weakly mutagenic (25), that its metabolites are known to interact with functional proteins (28) and that these

interactions are consistent with the observation of chromosomal effects *in vitro* (26,27), protein binding may be a more significant measure of bioreactive benzene metabolite formation *in vivo* than DNA binding (24,28). We are attempting to identify the specific protein targets of benzene in exposed animals at this time.

Finally, these data show that tissue available dose and liver protein adducts plateau above benzene doses of ~20 mg/kg in this mouse strain. Thus, either the metabolism of benzene to the reactive intermediates that bind protein in liver was saturated or the available reactive (nucleophilic) centers in the protein were fully occupied. This saturation was not seen in bone marrow. Similar results were obtained by Mazullo *et al.* (10) from Wistar rats, showing that hepatic DNA and protein adducts isolated 22 h post-exposure were not linear with dose at the highest benzene doses and that protein binding was saturated earlier than DNA binding. These results are consistent with the conclusion that protein represents a high affinity, low capacity binding site for benzene metabolites (30).

In summary, we have used the technique of accelerator mass spectrometry to follow [^{14}C]-benzene dosimetry and have found that benzene and its metabolites are rapidly absorbed and cleared in the $B_6C_3F_1$ mouse at low doses. We find that benzene's total dose to the tissues and the bioactive component of that dose (estimated from macromolecular adduct levels) will scale linearly with administered dose below doses of ~20 mg/kg body wt to doses in the low ng/kg body wt range. This dose–response range encompasses human environmental exposure levels and the doses used in rodent bioassays. Additionally, these data show that greater levels of bioactive benzene intermediates are present at low dose in the primary response organ for benzene toxicity, bone marrow, relative to a non-target tissue (liver), where significant amounts of metabolites are generated.

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