



Benzene Disposition in the Rat after Exposure by Inhalation'

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Received August 15, 1978; accepted March 7, 1979

Benzene Disposition in the Rat after Exposure by Inhalation. RICKERT, D. E., BAKER, T. S., BUS, J. S., BARROW, C. S., AND IRONS, R. D. (1979). *Toxicol. Appl. Pharmacol.* **49**, 417-423. Little information is available on benzene disposition after exposure by inhalation despite the importance of this route in man. Benzene metabolites as a group have been measured in bone marrow, but quantitation of individual metabolites in this target tissue has not been reported. Male Fischer-344 rats were exposed to 500 ppm benzene in air and the uptake and elimination was followed in several tissues. Concentrations of free phenol, catechol, and hydroquinone in blood and bone marrow were also measured. Steady-state concentrations of benzene (11.5, 37.0, and 164.0 $\mu\text{g/g}$ in blood, bone marrow, and fat, respectively) were achieved within 6 hr in all tissues studied. Benzene half-lives during the first 9 hr were similar in all tissues (0.8 hr). A plot of amount of benzene remaining to be excreted in the expired air was biphasic with $t_{1/2}$ values for the α and β phases of 0.7 and 13.1 hr, respectively. Phenol was the main metabolite in bone marrow at early times (peak concentration, 19.4 $\mu\text{g/g}$). Catechol and hydroquinone predominated later (peak concentrations, 13.0 and 70.4 $\mu\text{g/g}$, respectively). Concentrations of these two metabolites declined very slowly during the first 9 hr. These data indicate that free catechol and hydroquinone persist in bone marrow longer than benzene or free phenol.

The disposition of benzene in mammals has been studied for a number of years. The most complete set of data concerning benzene distribution and elimination after exposure by inhalation is that of Schrenk *et al.* (1941). These workers exposed dogs to benzene at concentrations ranging from 500 to 1300 ppm. Steady-state concentrations of benzene in the body were achieved rapidly (<2 hr); benzene concentrations were highest in bone marrow and adipose tissue, and benzene disappeared from the blood in a biphasic manner. Determination of the half-life of

benzene in blood was precluded by an insufficient number of data points, and quantitation of benzene metabolites in tissues was not attempted.

Benzene is converted to a number of metabolites in mammals. By 1949, the following compounds had been identified or suggested as urinary metabolites of benzene: phenol, catechol, hydroquinone, 1,2,4-trihydroxybenzene, *trans,trans*-muconic acid, phenylmercapturic acid, and dihydrocyclohexadiene (Porteus and Williams, 1949). Certain of these metabolites (phenol, catechol, hydroquinone) are much more toxic to bone marrow cultures than is benzene (Harrison and Randoll, 1948). Attempts to correlate benzene metabolism with benzene-induced

A preliminary report of this work was presented at the joint meeting of the American Society for Pharmacology and Therapeutics and the Society of Toxicology, August 1978, Houston, Tex.

myelotoxicity have produced some conflicting data (Ikeda, 1964; Ikeda and Ohtsuji, 1971; Snyder and Kocsis, 1975; Ikeda *et al.*, 1972), but the bulk of evidence suggests that a benzene metabolite is responsible for the myelotoxicity. This can be illustrated by the work of Andrews *et al.* (1972) who used depression of red cell ^{59}Fe uptake as an estimate of benzene-induced myelotoxicity. Their data suggest that ^3H benzene metabolism and toxicity can be altered by coadministration of toluene, which resulted in less depression of red cell ^{59}Fe uptake than did administration of benzene alone. This protective effect of toluene was associated with a decrease in non-toluene-extractable radioactivity (presumably benzene metabolites) in bone marrow.

It is clear from the foregoing that the rational design of experiments to assess hazards of benzene exposure in man and to determine the mechanism of benzene-induced myelotoxicity requires a more complete knowledge of benzene disposition after inhalation exposure. It is especially important that concentrations of benzene and its metabolites be measured individually in bone marrow. This report describes benzene distribution during and following exposure of Fischer-344 rats to 500 ppm benzene in air. In addition, three major benzene metabolites, phenol, catechol, and hydroquinone, were quantitated in the blood and bone marrow of the exposed animals.

METHODS

Animals and chemicals. Male Fischer-344 rats (Charles River Co., Wilmington, Mass.) weighing 200–250 g were used (three/group). For at least 1 week prior to benzene exposure, the animals were housed in a humidity- and temperature-controlled room and given food (Wayne Lab Blox, Allied Mills, Chicago, Ill.) and water *ad libitum*.

Benzene was obtained from Fisher Scientific Company (certified A.C.S., lot No. 770535). Methanol was obtained from Burdick and Jackson (Muskegon, Mich.). Standard phenol, catechol, hydroquinone, and d_6 -phenol and trifluoroacetic anhydride were obtained from Aldrich Chemical Company (Milwaukee,

Wisc.). Deuterated benzene (d_6) was Merck and Company, Inc. (Pointe Cl

Exposure procedure. Rats were exposed in two dynamic air flow chambers. The small chamber, operated at an air flow rate of 100 liters/min, was used to determine approach concentrations of benzene. The t_{90} (99% of nominal benzene concentration) in the small chamber was 23.9 min as calculated by Silver (1946). Exposure for elimination experiments was carried out in a 1100-liter glass steel chamber operated at an air flow rate of 100 liters/min ($t_{90} = 20.3$ min). Duration of exposure was measured from the time that benzene was begun. Benzene concentration in the small chamber was monitored by gas chromatography (Cow-Mac 750 Series FID) using a glass column (Tenax GC 60/80 mesh). In the large chamber, benzene concentration was monitored by infrared spectrometry (Wilk) at a wavelength of 3.214 μm . Concentrations in the small chamber ranged from 474 to 499 ppm (mean TWA = 10 ppm). Exposure of animals to 500 ppm for 6 hr in each chamber produced similar concentrations of benzene.

Sampling procedure. Animals were sacrificed by decapitation. In the experiments to approach steady state, animals were exposed for 0.5, 1, 1.5, 2, 4, 6, or 5 hr of elimination experiments, animals were exposed for 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, or 9 hr after exposure. Blood was collected in heparin. Tissue samples were removed in the following order: liver, lung, heart, spleen, adipose tissue from the peritoneal cavity, and bone marrow was removed from the femur. Samples were kept on ice at all times and processed (see below) immediately after exposure.

Sample treatment. One hundred microliters of blood containing heparin was mixed with 100 μl internal standard solution (1 μg methanol). Weighed samples of bone marrow were mixed with 100 μl internal standard solution. Samples were homogenized using a vortex mixer in 3 ml internal standard solution. Particulate matter was removed by centrifugation at 4000 rpm for 15 min. The supernate were analyzed by selected ion monitoring (see below). The practice of chilling samples as soon as possible with ice minimized benzene loss, yielding 95% of those expected in preliminary

The benzene metabolites, phenol

hydroquinone, were analyzed as the trifluoroacetate derivatives. Five milliliters of methanol and 100 μ l of internal standard solution (1 μ g d_6 -phenol/ml methanol) were added to 1 ml of blood, weighed samples of bone marrow or standard solutions of phenol, catechol, and hydroquinone. Particulate matter was removed by centrifugation and 100 μ l of 0.1 M KOH in methanol was added to the supernate. The samples were dried under nitrogen and reconstituted in 100 μ l trifluoroacetic anhydride for analysis by selected ion monitoring (see below).

Analysis of samples. Analyses were performed on a Finnigan 4023 gas chromatograph-mass spectrometer with an IncoS data system. The following conditions were held constant throughout: helium carrier

gas at a flow of 20 ml/min; temperature of the injection port, 200°C; temperature of the glass-jet separator and transfer lines, 240°C; ionization voltage, 70 eV; emission current, 500 μ A; electron multiplier voltage, 1675 V.

For the analysis of free benzene the samples were separated on a 6 ft $\times$ 2-mm i.d. glass column (Tenax GC, 60/80 mesh) temperature programmed from 190 to 225°C at a rate of 24°C/min. Selective ion monitoring (SIM) was used to monitor ions m/e 78 for benzene and m/e 84 for d_6 -benzene. For quantitative analysis, standard curves were prepared in methanol ranging in concentration from 100 pg/ μ l to 100 ng/ μ l holding the concentration of the internal standard at 1 ng/ μ l. Ratios of peak areas at m/e 78

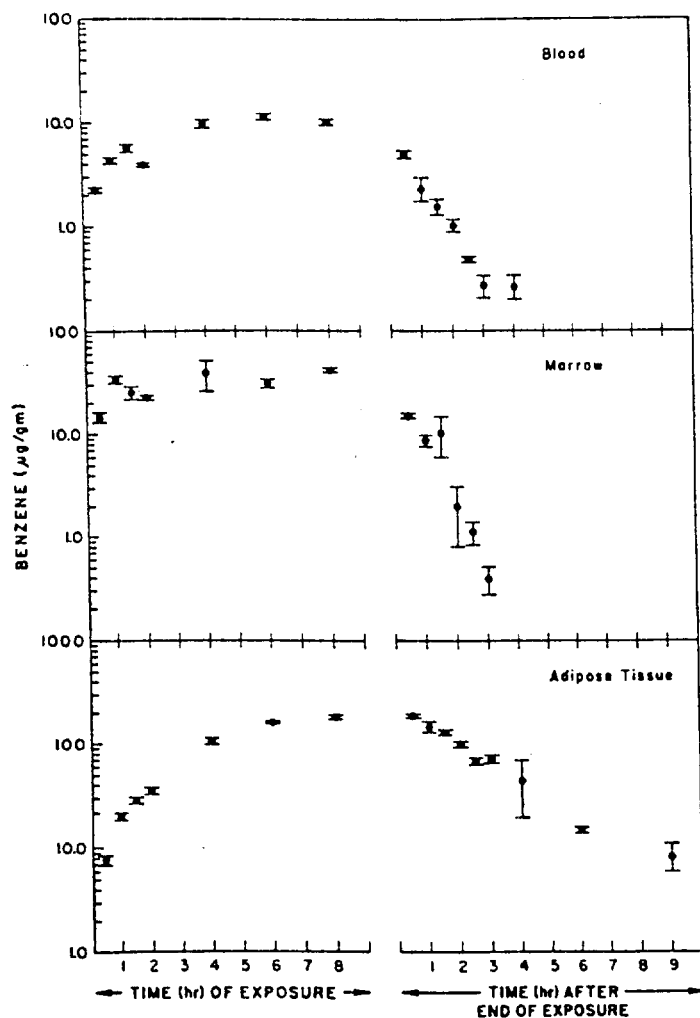


FIG. 1. Benzene concentration in blood, bone marrow, and adipose tissue during and following inhalation exposure. Each point is the mean $\pm$ SE for three animals. Points on the decay phase were obtained after a single 6-hr exposure.

and *m/e* 84 were calculated and plotted against nanograms of benzene per microliter. These standard curves were used to calculate the concentrations of benzene in blood, bone marrow, and tissues.

Concentrations of benzene eliminated in expired air were measured in three animals. Immediately after a 6-hr exposure to 500 ppm benzene, animals were removed to all-glass metabolism units (one rat/unit). Air flow was adjusted to 400 ml/min. Benzene was trapped on 1 g activated coconut charcoal (6-14 mesh, Fischer Scientific Co.) in 5-ml disposable pipets. Traps were changed at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 9.0, 12.0, 24.0, 36.0, and 48.0 hr after introduction of the rat into the unit. The benzene was eluted from the traps with carbon disulfide; the charcoal was transferred to 5-ml amber reaction vials, and 3 ml CS₂ (Fisher A.C.S. Reagent) was injected through a septum fitted over the vials. The CS₂ was then injected into a gas chromatograph with a flame ionization detector (Gow-Mac, Series 750) under the following conditions: 6 ft x 2-mm i.d. glass column (Tenax GC 60/80 mesh) held at 135°C. The carrier gas was nitrogen at a flow rate of 30 ml/min. The temperatures of the injection port and detector were held at 165°C. Standard curves of benzene in CS₂ were prepared ranging in concentration from 0.5 to 5 µl/ml and plotted as concentration benzene vs peak height. In a preliminary experiment, this method of trapping and eluting benzene was shown to result in a recovery of >99%. Percentage of benzene remaining to be excreted in the expired air was calculated for each time interval for each animal by dividing the amount of benzene eliminated during the time interval by the total amount eliminated in expired air.

The trifluoroacetate derivatives of phenol, catechol and hydroquinone were analyzed by gc/ms. The compounds were separated on a 6 ft x 2-mm i.d. glass column (3% OV1 on 60/80 mesh DMCS Chromasorb W) held at 90°C. SIM was used, monitoring the derivative of the internal standard (*d*₅-phenol) at *m/e* 195; phenol at *m/e* 190; catechol and hydroquinone at *m/e* 302. Standard solutions (50 pg/µl to 50 ng/µl) of phenol, catechol, and hydroquinone were prepared in methanol. The concentration of the internal standard was held at 1 ng/µl. Peak area ratios (compound/*d*₅-phenol) were calculated for each compound and plotted against nanograms per microliter.

RESULTS

Benzene concentrations reached steady state within 4 hr in blood, 6 hr in fat, and less than 2 hr in bone marrow (Fig. 1). Steady-state benzene concentrations and half-times for approach to steady state and

TABLE I
STEADY-STATE BENZENE CONCENTRATIONS AFTER
EXPOSURE OF RATS TO 500 ppm BENZENE FOR 6 hr
AND HALF-TIME FOR UPTAKE AND ELIMINATION OF
BENZENE.

Sample	Steady-state concentration (µg/g or /ml)	Half-times (hr in hr)	
		Approach to steady- state ^a	Elimina- tion ^b
Blood	11.5 ± 0.7	1.4	0.7
Bone marrow	37.0 ± 2.2	— ^c	0.5
Fat	164.42 ± 15.0	2.0	1.6
Liver	9.9 ± 0.7	1.9	0.4
Lung	15.1 ± 0.9	1.5	0.4
Kidney	25.32 ± 1.3	1.3	0.6
Spleen	4.92 ± 0.5	0.9	0.8
Brain ^d	6.5 ± 0.6	2.6	0.6

^a N = 3. Data are expressed as means ± SE.

^b Determined graphically from data points at 0.54 hr after beginning of exposure.

^c Determined graphically from data points at 0.5-9 hr after end of exposure.

^d Steady-state approached too rapidly for determination of half-time.

elimination are summarized in Table I. This table indicates the similarity between elimination half-lives for all tissues except fat.

Phenol was present in both blood and bone marrow after a 6-hr exposure to benzene (Fig. 2), but it rapidly disappeared. Catechol and hydroquinone concentrations remained fairly constant over the time period studied.

Benzene was eliminated in the expired air in a biphasic manner (Fig. 3). The two components of the curve were determined graphically for each of the three animals exposed and the coefficients were averaged. The equation found to represent the curve was $y = 93e^{-0.007t} + 4.93e^{-0.354t}$. The first component (line A) has a half-life of 0.7 hr. and the second component (line B) has a half-life of 13.1 hr.

DISCUSSION

Data collected here essentially confirm the information obtained in dogs with regard to relative concentrations of benzene in various

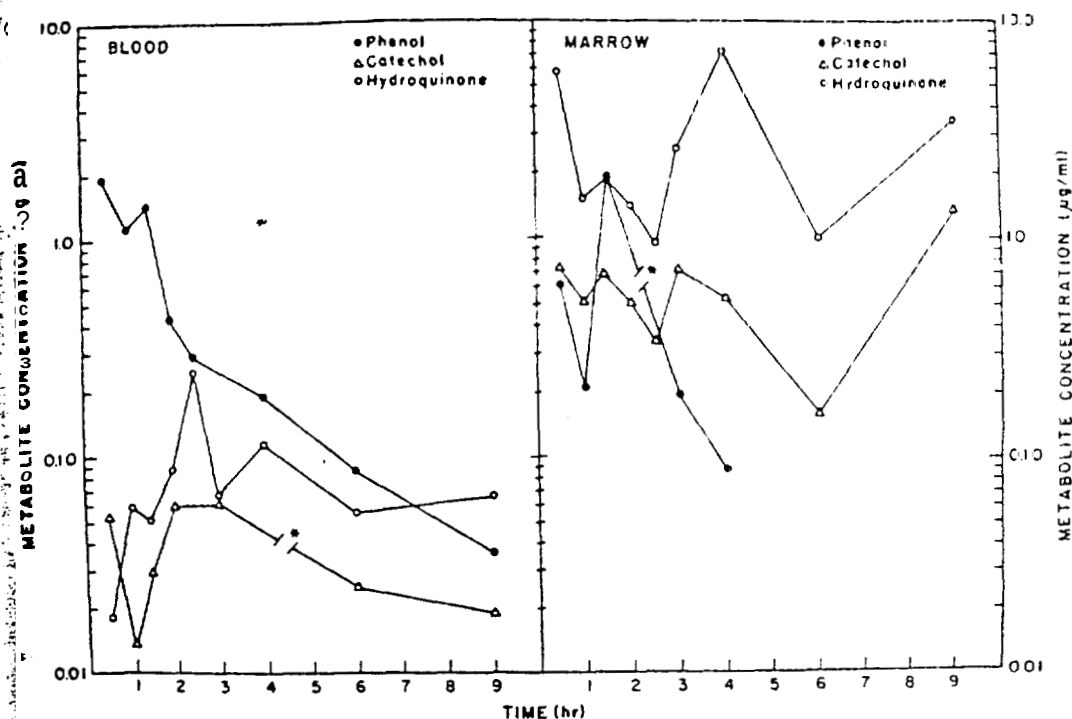


FIG. 2. Concentration of metabolites following a 6-hr inhalation exposure to benzene. Asterisks indicate points at which a compound was not determined or not detected due to technical difficulties. SE were all less than 50%. Each point is the mean for three animals.

tissues (Shrenk *et al.*, 1941). Bone marrow, adipose tissue, and kidney contained concentrations of benzene in excess of that in blood. This is probably due to the lipophilic nature of benzene. Half-times for achieving steady-state were of the same order for all tissues except bone marrow, and they appear to be somewhat longer than those found in dogs (Shrenk *et al.*, 1941). The reasons for this species difference could include differences in fraction absorbed or differences in rates of excretion or metabolism. The data on benzene approach to steady-state indicate that a commonly used protocol for inhalation exposure (6 hr/day) would result (at least for rats) in maintenance of steady-state concentrations of benzene for about 2 hr.

The most complete description of benzene elimination obtained in this study was that for benzene excretion in the expired air. The elimination curve is clearly biphasic, suggesting that benzene elimination can be des-

cribed mathematically by a two-compartment model. The half-time for the more rapid phase of elimination in expired air agrees well with the half-times calculated for disappearance from various tissues (0.7 vs 0.4–1.6 hr). The assay for benzene in tissues was not sufficiently sensitive to describe the slow phase of benzene elimination from tissues. The half-life for the slow phase of benzene elimination in expired air (13.1 hr) suggests that some accumulation of benzene could occur under this regimen of exposure. The maximum benzene concentration in blood which could be achieved can be approximated by:

$$C_{\infty} = C_1 \left[1 - \exp \left(-\frac{0.693}{t_{1/2}} \Delta t \right) \right]^{-1},$$

where C_1 is the concentration achieved during a single exposure, $t_{1/2} = 13.1$, and Δt is the interval between exposures (24 hr).

Substituting the actual values yields a

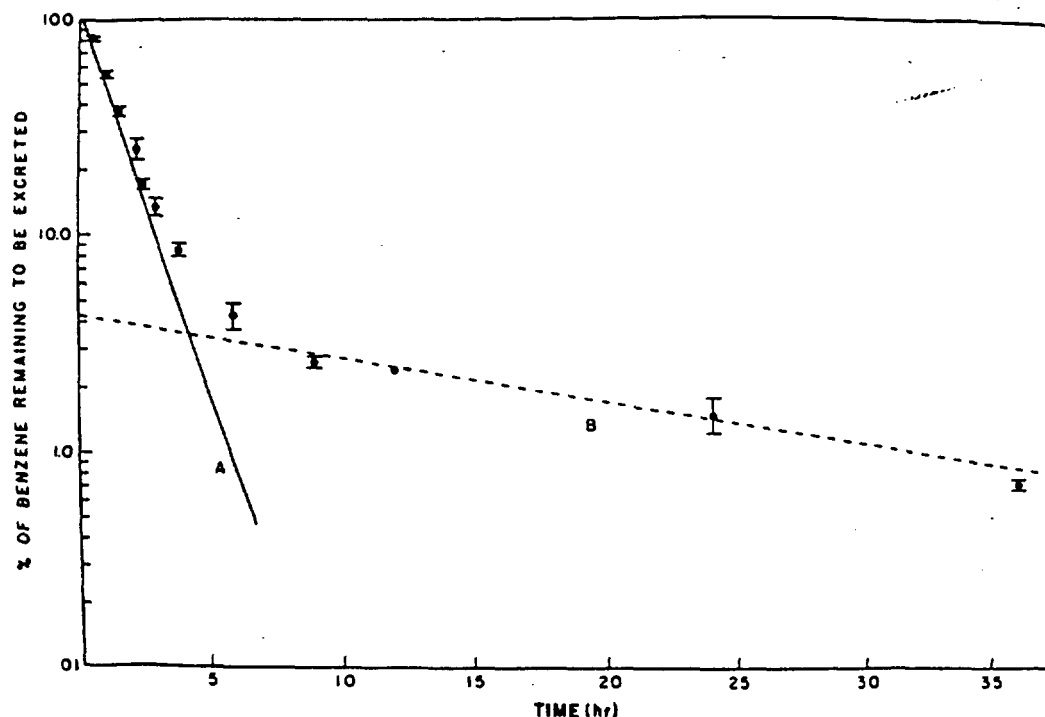


FIG. 3. Percentage of benzene remaining to be excreted in expired air. Each point is the mean $\pm$ SE for three animals with the exception of the 12-hr time point which represents only a single value.

blood concentration of $16 \mu\text{g/ml}$ after an infinite number of exposures. One may also predict that after four daily exposures, blood concentrations of benzene will be $15.9 \mu\text{g/ml}$. This agrees very well with the experimental value found ($15.5 \pm 1.6 \mu\text{g/ml}$) in preliminary experiments.

Free phenol, catechol, and hydroquinone were all found in blood and bone marrow following 6 hr of exposure to benzene. The concentrations of free phenol in blood and bone marrow decreased much more rapidly after exposure ceased than did those of free catechol or hydroquinone. It is interesting that both catechol and hydroquinone persisted in blood and bone marrow for at least 9 hr after exposure to benzene stopped. This suggests a possibility for accumulation of these compounds. Catechol and hydroquinone are 4-16 times more toxic to bone marrow cultures than phenol or certain trihydroxybenzenes (Harrison and Randall,

1948), but their ability to produce myelotoxicity after administration to intact rats has not been established. Mitchell (1971) was unable to produce aplastic anemia in rats at doses of phenol, catechol, or hydroquinone which produced significant lethality, but benzene itself caused the disorder. Nomiyama (1965) used much lower doses of phenol, catechol, hydroquinone, and other benzene metabolites to treat rats. He demonstrated that catechol, given at a dose of 30 mg/kg/day for 7 days, produced a decrease in peripheral leukocyte count similar to that seen when benzene was administered. With the demonstration, in this study, of the possibility for accumulation of catechol and hydroquinone in bone marrow of rats receiving benzene, it seems necessary to investigate further the role of these compounds in benzene-induced myelotoxicity. The assay procedure developed here should allow correlation of individual benzene metabolites

in bone marrow with the toxic effects observed. The hypothesis that catechol or hydroquinone (or both) is the causative agent is attractive because it requires neither the transport of a highly reactive intermediate (e.g., benzene oxide) from liver to bone marrow nor the demonstration of an active mixed function oxidase system in bone marrow.

ACKNOWLEDGMENTS

The authors thank C. Ramaley and D. Deyo for assistance in the quantitation of benzene in expired air.

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