

BENZENE IS METABOLIZED AND COVALENTLY BOUND IN BONE MARROW IN SITU

R.D. IRONS, J.G. DENT, T.S. BAKER and D.E. RICKERT

*Chemical Industry Institute of Toxicology, P.O. Box 12137, Research Triangle Park,
NC 27709 (U.S.A.)*

(Received July 2nd, 1979)

(Revision received December 10th, 1979)

(Accepted December 14th, 1979)

Introduction

Benzene is a well-known myelotoxic agent which has recently been implicated as a leukemogen [1]. The mechanism by which benzene induces myelotoxicity is not understood. Myelotoxicity could result from the action of the parent compound or any of the well-known metabolites of benzene such as phenol, catechol, hydroquinone, 1,2,4-benzenetriol or *trans,trans*-muconic acid [2–4]. Quantitatively, the liver is the major site of benzene metabolism in the body [5]; however, the relative importance of metabolism in the liver with respect of myelotoxic action is unknown. The intermediate(s) responsible for bone marrow toxicity may be synthesized in the liver and transported to marrow where they act directly; they may require additional metabolism in bone marrow to form the ultimate toxic intermediate; or, as an alternative, bone marrow metabolism of benzene may be the only prerequisite for benzene induced myelotoxicity. The possibility that benzene can be metabolized directly in bone marrow, the principal target organ, has not been explored [6]. Phenol, catechol and hydroquinone are found in rat blood and bone marrow following inhalation of benzene [7], but the sites of formation of these metabolites have not been established, nor has the metabolic contribution of the bone marrow been ascertained. The principal objective of this work was to determine if bone marrow is capable of metabolizing benzene independent of metabolism in the liver.

Materials and methods

Male Fischer-344 rats (Charles River, Wilmington, MA), weighing 250–350 g, were subjected to a hind limb perfusion procedure. The left common iliac vein and artery were cannulated in rats maintained under anileridine-ketamine anesthesia. The isolated limb was then perfused at a flow rate of $1 \text{ ml} \cdot \text{min}^{-1}$ with citrated oxygenated whole rat blood maintained at 39°C . A single dose (0.5–2 mCi) of [^{14}C]benzene (Midwest Research Institute, Kansas City, MO) ($39 \text{ mCi} \cdot \text{mmol}^{-1}$) was introduced directly into the bone marrow space through a 0.75 mm hole drilled in

the distal head of the femur. The bone was immediately sealed with bone wax. The total volume of benzene administered ranged between 1 and 3 μ l. In control preparations the bone marrow tissue was removed by aspiration prior to the introduction of radiolabelled benzene.

Blood was collected from the iliac vein in 10-min fractions for 1 h. Each 10-min fraction averaged 6.5 ml blood. Following the perfusion, collected blood samples and bone marrow were spiked with carrier compounds (50 μ g each of phenol, catechol, hydroquinone, 1,2,4-benzene triol and *trans,trans*-muconic acid) and acidified to a pH of 1.0 with 1 M HCl. Samples were then extracted with ethylacetate, dried over magnesium sulfate and filtered using Millipore Clarification Kits (Millipore Co., Bedford, MA). Extracts were concentrated under nitrogen and passed through silica gel. Final samples were concentrated to 20 μ l and subjected to high pressure liquid chromatographic analysis (HPLC).

HPLC analysis was carried out using a Waters system (Milford, MA) equipped with two Zorbax silica columns (Dupont, Wilmington, DE), flow programmed from 0.8–1.6 ml $\cdot$ min⁻¹. The solvent system was 74.9% methylene chloride, 25% ethylacetate, 0.1% methanol with 100 μ l of 88% formic acid added per 100 ml of solvent. Fractions were collected at 0.3 min intervals starting 9 min after injection and analyzed for radioactivity by liquid scintillation.

Total metabolites bound in bone marrow were determined by subjecting the ethylacetate extracted bone marrow residue to further extraction with 5% trichloroacetic acid (TAC) and increasing concentrations of aqueous methanol (40, 60, 80 and 100%). The solvent concentration was changed when no radioactivity was detected above background in the supernates, each sample subjected to an average of 17 extractions. Find residues were solubilized in a mixture of NaOH, methanol and Triton-X-100 and analyzed for radioactivity by liquid scintillation.

Results and discussion

A representative profile for benzene metabolites obtained by HPLC analysis is presented in Fig. 1. Radioactivity was found to co-elute with phenol, catechol, and hydroquinone in blood perfusate and bone marrow fractions. No radioactivity co-eluted with metabolites in blood spiked with radioactive benzene and extracted immediately. Peak levels for all three metabolites in blood were found in the 10-min fraction (Fig. 2) with total radioactive metabolites recovered averaging 6.44 nmol ($\pm$ 1.2, S.E. n = 4) after 1-h incubation. The same radioactive metabolites were recovered from bone marrow (phenol, 64 ± 23 ; catechol, 3.9 ± 1.6 ; and hydroquinone, 0.31 ± 0.2 pmol $\cdot$ mg⁻¹). No radioactive metabolites were detected in Control preparations and no radioactivity was detected in systemic blood, liver or urine in any of the perfusion experiments. Thus the small amount of metabolism observed is directly attributable to the bone marrow.

Covalent binding of benzene or its metabolites was investigated using the extraction procedure described. Results are presented in Table I. Total

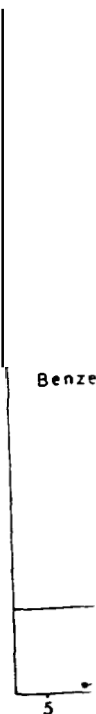


Fig. 1. Radioactivity (254 u)

radioactivity recovered and followed by metabolite binding to liver and bone marrow. The

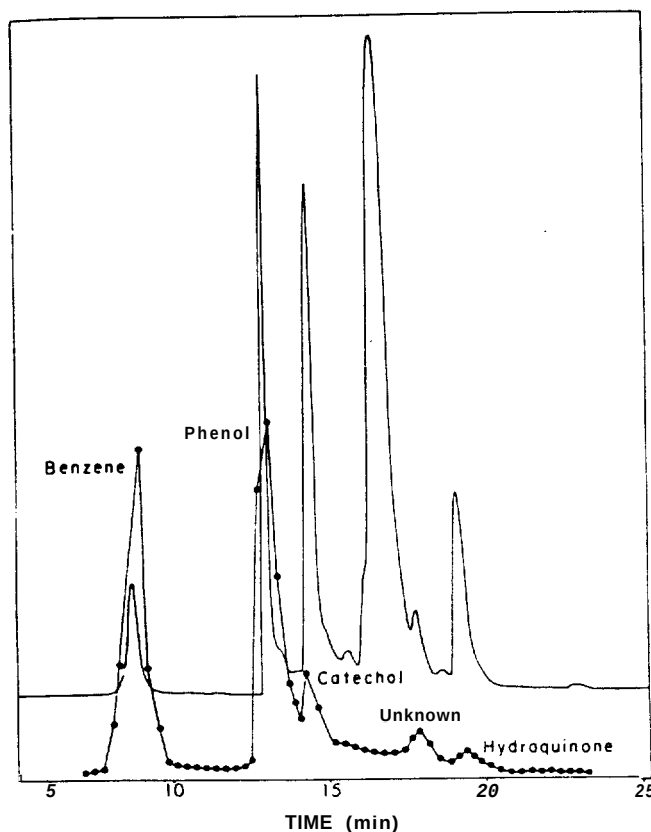


Fig. 1. Analysis of benzene metabolites by HPLC. Absorbance of 254 nm (—). Radioactivity (—●—●—). Fractions were collected at 0.3 min intervals starting 7 min. after injection of the sample. Total time for sample elution: 25 min. —, UV absorbance (254 nm); —●—●—, radioactivity (cpm).

radioactivity bound in the bone marrow represents 14.7% of the metabolites recovered from the bone marrow or 2.76% of the total metabolites in blood and bone marrow. Addition of radiolabelled benzene to bone marrow in vitro followed by immediate extraction resulted in retention of 11 pmol equivalents versus an average of 239 pmol in perfusion experiments. Thus, metabolism of benzene appears to be a necessary prerequisite for covalent binding in bone marrow.

The results of this study clearly establish the capability of bone marrow to metabolize benzene independent of metabolism of the compound by the liver. However, the amount of metabolism appears to be very small, recovered metabolites representing only 2×10^{-4} of 1% of the administered dose. It is impossible to assess in physiologic terms the contribution of bone marrow to overall benzene metabolism under these experimental conditions. The initial levels of benzene in bone marrow in these experiments

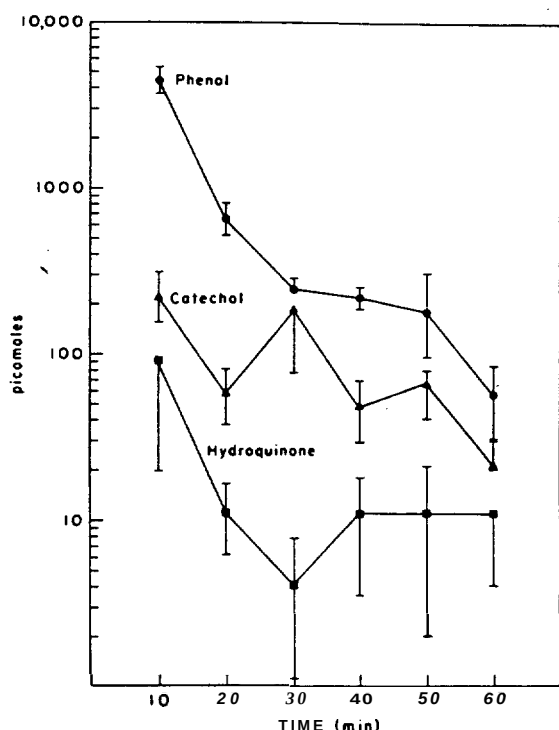


Fig. 2. Radioactive metabolites recovered from blood following the introduction of [^{14}C]benzene directly into bone marrow.

(94–195 $\mu\text{g} \cdot \text{mg}^{-1}$) are higher than are likely to be encountered as a consequence of exposure of the whole animal [7]. In addition, the perfusion procedure provides for an examination of only a first pass effect with no recirculation of metabolites or parent compound. It was impossible to measure accurately the recovery of radioactive benzene due to the extraction procedure employed; however, it is likely that benzene rapidly enters the

TABLE I

BENZENE METABOLISM AND COVALENT BINDING IN BONE MARROW

Total metabolites recovered from blood and bone marrow	Metabolites recovered from blood	Metabolites recovered from bone marrow	Metabolites bound in bone marrow
8672 ^a ± 1537 ^b	6441 ± 1242	1626 ± 661	239 ± 107

^a Metabolites are expressed as picomoles and bound metabolites as picomole equivalents of benzene.

^b The data are expressed as mean ± standard error from five bone marrow and four blood perfusate fractions.

perfusate
benzene

Althou
only 25%
(mean W
bolites in
marrow.
rapidly E
bolites in
of the
exposure
concentr.
declined
blood ap
experime
bolites fo

We tha
cal assist

- 1 S. Lask
Environ
- 2 J.W. PC
benzene
imide. (receiving
quinol a
- 3 D.V. P
benzene
- 4 D.V. Pa
phenylg
of [^{14}C]
- 5 R. Snyder
C.R.C. (288.
- 6 R. Snyder
liver and
- 7 D.E. Ri
rats after

perfusate and thus the marrow was only exposed to a high concentration of benzene for a short time.

Although the total metabolites recovered from bone marrow represent only 25% of those in blood, the concentration of metabolites in marrow (mean weight = 20 mg) is much higher. The concentration of total metabolites in blood is $0.215 \text{ nmol} \cdot \text{g}^{-1}$ as compared to $81.3 \text{ nmol} \cdot \text{g}^{-1}$ for bone marrow. The fact that the appearance of metabolites in blood diminishes rapidly after 10 min demonstrates a high retention of water soluble metabolites in bone marrow. This finding is in keeping with the results of studies of the disposition of benzene metabolites in rats following inhalation exposure (6 h) to benzene (500 ppm) in which catechol and hydroquinone concentrations persisted in bone marrow long after blood levels had declined [7]. The metabolite concentration ratio between bone marrow and blood approaches 400 in this study as compared to 7 in the inhalation experiment, supporting the conclusion that a high proportion of the metabolites formed in bone marrow is retained in that tissue.

We thank B.J. Moore, C.N. Smith and D.H. Williams whose expert technical assistance made this work possible.

- 1 S. Laskin and B.D. Goldstein, Benzene toxicity: a critical evaluation, *J. Toxicol. Environ. Health*, Suppl. 2 (1977).
- 2 J.W. Porteus and R.T. Williams, Studies in detoxification 19. The metabolism of benzene. 1. (a) The determination of phenol in urine with 2:6-dichloroquinonechloroimide. (b) The excretion of phenol glucuronic acid, and ethereal sulphate by rabbits receiving benzene and phenol. (c) Observations on the determination of catechol, quinol and muconic acid in urine, *Biochem. J.*, 44 (1949)56-61.
- 3 D.V. Parke and R.T. Williams, Studies in detoxification 49. The metabolism of benzene containing [^{14}C]benzene, *Biochem. J.*, 54 (1953)231-238.
- 4 D.V. Parke and R.T. Williams, The metabolism of benzene (a) The formation of phenylglucuronide and phenylsulphuric acid from [^{14}C]benzene. (b) The metabolism of [^{14}C]phenol, *Biochem. J.*, 55 (1953)337.
- 5 R. Snyder and J.J. Kocsis, Current concepts of benzene toxicity, in: L. Golberg (Ed.), *CRC. Critical Reviews in Toxicology*, CRC. Press, Cleveland, Ohio, 1975, pp. 265-288.
- 6 R. Snyder, E.W. Lee and J.J. Kocsis, Binding of labeled benzene metabolites to mouse liver and bone marrow, *Res. Comm. Chem. Path. Pharmacol.*, 20 (1978)191.
- 7 DE. Rickert, T.S. Edgar, C.S. Barrow, J. BUS and R.D. Irons, Benzene disposition in rats after inhalation exposure, *Toxicol. Appl. Pharmacol.*, 49 (1979)417.

ction of

ed as a
rfusion
with no
ibie to
raction
ers the

olites
in bone

.07

ivalents

ir blood