Analytical Approaches to the Study of the Disposition of Myelotoxic Agents

by Douglas E. Rickert,* Terrie S. Baker,* and John P. Chism*

A correlation of myelotoxic effect with concentration or a foreign compound of its metabolite at the site of action may provide useful insights into the mechanism of toxic action and/or its amelioration. This correlation requires sensitive and specific assay methods. This communication describes useful methods for the study of benzene disposition in rodents. The assays are sensitive, specific, and rapid. They rely on gas chromatography-mass spectrometry and on high performance liquid chromatography. These methods have allowed measurement of catechol, phenol, and hydroquinone in samples of rodent bone marrow following inhalation exposure to benzene. Their application to the study of benzene metabolism in rat bone marrow in situ is also described.

Introduction

The study of myelotoxicity, like the study of other forms toxicity, requires a thorough knowledge of the disposition of the toxicant under investigation. The ultimate goal is the identification of the proximal toxicant and a description of the interactions of that toxicant with cellular constituents of the target issue. Properly designed disposition studies can yield useful information on the structure and concentration of putative proximal toxicants at the site of action. In this respect, disposition studies of myelotoxic agents are similar to disposition studies of any other type of toxic agent.

Analysis for myelotoxic agents and their metabolites in the target tissue does, however, present some interesting problems. The scarcity of bone marrow tissue in rodents requires extremely efficient and sensitive methods for analysis. The studies described here were done with benzene. In addition to problems associated with sampling and analysis of small amounts of tissue, benzene and its metabolites pose analytical problems because of their volatility, instability, and/or highly polar nature.

Successful circumvention of some of these problems has been accomplished with combined gas chromatography-mass spectrometry and high performance liquid chromatography. We have used these techniques to describe the pharmacokinetics of benzene after inhalation exposure in rats (1) to demonstrate that bone marrow has metabolic activity toward benzene (2), and to begin to correlate benzene metabolism in vitro with alterations in vivo benzene toxicity produced by pretreatment with other foreign compounds (3).

Methods and Results

Male, Fischer-344 rats (200-250 g) were exposed to 500 ppm benzene in a 1100 liter dynamic air flow chamber for 6 hr. The time required for benzene concentration to reach 99% of the target concentration was 20.3 min. At the end of the exposure period the animals were removed. Groups of three were sacrificed by decapitation 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, and 9 hr after removal. Heparinized blood, perirenal fat, and bone marrow were analyzed for benzene. The goals in developing a method for benzene in these tissues included sufficient sensitivity to measure benzene in bone marrow, specificity sufficient to ensure that endogenous compounds did not interfere with the measurement, and a minimi-
zation of sample preparation so that the volatility of benzene would not lead to erroneously low values.

In order to correct for the volatility of benzene, deuterated (d₆) benzene (Merck and Co., Inc., Quebec, Canada) was added as an internal standard immediately after removal of the tissues. Methanol (0.1 ml) containing 1 µg benzene-d₆/ml was added to 0.1 ml blood. A 15 to 25 mg portion of bone marrow was mixed with 0.1 ml methanol containing 1 µg benzene-d₆/ml. All other tissues were homogenized on ice in 3 ml methanol (containing 1 µg benzene-d₆/ml) of wet weight tissue. All samples were sonicated and mixed then centrifuged at 1500g for 15 min. Then 1-5 µl of the supernate was injected onto the gas chromatograph-mass spectrometer (Finnegan 4021, Finnegan Corp., Sunnyvale, Calif.). The mass spectrometer was operated in the selected ion monitoring mode and alternately focused on m/e 78 (parent ion of benzene) and m/e 84 (parent ion of benzene-d₆). Reconstructed mass chromatograms of a typical standard are shown in Figure 1. The gas chromatography column was Tenax GC (Suppleco, Inc., Bellafonte, Pa.) packed in a 2 m × 2 mm (i.d.) glass column. Helium carrier gas was admitted to the column at 20 ml/min. The column was temperature programmed from 190 to 225°C at a rate of 24°C/min. The ratio of the area under the peak at m/e 78 to the area under the peak at m/e 84 was plotted against the concentration of benzene in the standards (Fig. 2). The standard curve was linear over final benzene concentrations of 100 ng/ml to 100 µg/ml. Thus, the method allowed quantitation of benzene at concentrations between 200 ng/ml and 200 µg/ml in blood, between 200 ng/g and 200 µg/g in bone marrow (if volumes injected onto gas chromatography-mass spectrometer were increased to 5 µl), and between 400 ng/g and 400 µg/g in other tissues. Disappearance of benzene from blood, bone marrow, and perirenal fat is shown in Figure 3.

Three metabolites of benzene, phenol, catechol, and hydroquinone, were measured in blood and bone marrow from the above rats. A 5-ml portion of methanol was added to 1.0 ml blood or 15-25 mg bone marrow. Methanol (0.1 ml) containing 1.0 µg phenol-d₆/ml (Merck & Co., Quebec, Canada) was added to the mixture which was then mixed vigorously and centrifuged at 1500g for 15 min. The supernate was transferred to a clean tube and 0.1 ml of 0.1M KOH was added. The samples were evaporated to dryness under a stream of N₂. Trifluoroacetic anhydride (Aldrich Chemical Co., Milwaukee, Wisc.) (500-100 µl) was added, and aliquots of 1-2 µl were injected onto the gas chromatograph-mass spectrometer. The resulting trifluoroacetate derivatives were separated on a 2 m × 2.0 mm i.d. glass column packed with 3% OV-1 on Chromasorb W (Supelco, Inc., Bellafonte, Pa.) and held at 90°C. The carrier gas was helium at a flow rate of 20 ml/min. The ions monitored by the mass spectrometer were m/e 195, the parent ion for fluorooacetylated phenol-d₆, one deuterium atom is lost in the derivatization reaction; m/e 190, the parent ion for fluorooacetylated phenol and m/e 302, the parent ion for fluorooacetylated catechol and hydroquinone. A typical mass chromatogram for a standard mixture is shown in Figure 4.

Environmental Health Perspectives
A standard curve was prepared by carrying 500 pg to 10 ng of phenol, catechol, or hydroquinone through the above procedure. A standard curve is given in Figure 5. Sensitivities were 50 ng of metabolite/g bone marrow or 500 pg of metabolite/ml blood. Concentrations of phenol, catechol, and hydroquinone at several times following the end of exposure and given in Table 1.

Both normal-phase and reversed-phase high performance liquid chromatography have been successfully applied to studies of benzene metabolism. There is evidence in the literature that rat bone marrow is metabolically competent toward xenobiotics (4), but there has only recently been a demonstration of bone marrow’s ability to metabolize benzene in situ (2). Because certain benzene metabolites, namely, sulfate and glucuronide conjugates, are not readily analyzed by combined gas chromatography-mass spectrometry, we employed normal-phase

![Figure 3. Disappearance of benzene from blood, bone marrow, and adipose tissue following 6 hr exposure to 500 ppm benzene in air.](image)

![Figure 4. Ion chromatograms of (top) fluoroacetylated phenol, (middle) phenol-d₅, (bottom, first peak) catechol and (bottom, second peak) hydroquinone. Numbers above peaks as in Figure 1.](image)

Table 1. Concentration of phenol, catechol, and hydroquinone in rat blood and bone marrow after a 6 hr exposure to 500 ppm benzene in air.

| Time from end of exposure, hr | Blood, ng/ml | Bone marrow, ng/g | Blood, ng/ml | Bone marrow, ng/g | Blood, ng/ml | Bone marrow, ng/g |
|------------------------------|--------------|------------------|--------------|------------------|--------------|------------------|
| 0.5                          | 1958 ± 540   | 6101 ± 1500      | 53 ± 23      | 7215 ± 704       | 18 ± 8       | 58480 ± 3037     |
| 1.0                          | 1108 ± 236   | 2808 ± 1600      | 14 ± 6       | 5087 ± 1586      | 58 ± 11      | 13010 ± 4320     |
| 1.5                          | 1431 ± 521   | 19410 ± 8517     | 30 ± 12      | 6982 ± 3633      | 50 ± 20      | 19150 ± 1070     |
| 2.0                          | 444 ± 64     | ND               | 59 ± 10      | 5033 ± 1027      | 89 ± 20      | 14030 ± 2790     |
| 2.5                          | 292 ± 35     | ND               | 72 ± 10      | 3476 ± 266       | 253 ± 100    | 9545 ± 811       |
| 3.0                          | 243 ± 61     | 1918 ± 1565      | 61 ± 30      | 7223 ± 1725      | 68 ± 9       | 26630 ± 5911     |
| 4.0                          | 197 ± 76     | 894 ± 730        | ND           | 5393 ± 1115      | 102 ± 35     | 42200 ± 18130    |
| 6.0                          | 62 ± 25      | ND               | 37 ± 12      | 2614 ± 850       | 52 ± 20      | 7050 ± 2150      |
| 9.0                          | ND           | ND               | 19 ± 12      | 13160 ± 4096     | 66 ± 30      | 35380 ± 1042     |

*Values are means of 3-6 rats ± S.E.M.

June 1981
high performance liquid chromatography. Male, Fischer-344 rats were aesthetized with an 
alieridine-ketamine mixture; the left common iliac vein and artery were cannulated and the isolated 
hind limb was perfused with citrated, oxygenated rat blood at a flow of 1 ml/min. Between 0.5 and 2.0 
mCi of $^{14}$C-benzene (39 mCi/m mole) was introduced directly into the marrow via a hole drilled at the 
distal end of the femur. Bone wax was used to close the hole, and blood was collected from the iliac vein 
for 1 hr in 10-min fractions. The bone marrow was removed at the end of the hour perfusion, and blood 
and bone marrow were analyzed for benzene metabolites. Blood (10 ml) or bone marrow (15-25 mg) 
was mixed with 50 µg each phenol, catechol, hydroquinone, and 1,2,4-benzenetriol. The pH was 
adjusted to 1.0 with 1.0M HCl, and the mixture was extracted three times with two volumes of 
ethyl acetate. The ethyl acetate layers were com-

FIGURE 5. Typical standard curves for phenol, catechol, and hydroquinone.

FIGURE 6. Normal-phase high pressure liquid chromatogram of benzene metabolites. See text for conditions.
resulting mixture was centrifuged at 2000g for 5 min, and 100-200 μl of the supernate was injected onto the high-pressure liquid chromatograph. The column used was octadecylsilic (Waters Radial Pak A) or octylsilane (Merck RP-8). Compounds were eluted in 20 min by a linear gradient from 100% water containing 100 μl formic acid/l to 100% methanol containing 100 μl formic acid/l. A typical elution profile of benzene metabolites is shown in Figure 7.

A first application of the new analytical procedure has been the study of benzene metabolism in vitro by various tissues. When hepatic 10,000g supernate was incubated in the presence of 14C-benzene and an NADPH regenerating system, two metabolite peaks appeared in the high performance liquid chromatogram. These peaks co-eluted with hydroquinone and phenol. When the same incubation was repeated with the addition of ATP, sodium sulfate, and uridine diphosphoglucuronic acid, the peaks co-eluting with hydroquinone and phenol decreased in size. An early-eluting peak, presumably a conjugate, appeared. Work to characterize fully the metabolites formed from benzene in vitro is continuing.

**Discussion**

Several methods for studying the disposition of benzene have been described. Each has its particular advantages and disadvantages, and each could be modified to study the disposition of other myelotoxic agents as well. The mass spectrometric methods described have the disadvantage of requiring expensive equipment and trained operators, but their sensitivity, specificity and simplicity of sample preparation make them very attractive. Using these methods we were able to show that half-lives for benzene in all tissues studied (except perirenal fat) were similar and that benzene is concentrated in bone marrow and fat as well as in organs of metabolism and excretion, the liver and kidney. In addition, concentration of phenol in blood and bone marrow declined rapidly after termination of exposure, but catechol and hydroquinone did not. This suggests a possibility of accumulation of these two potentially myelotoxic metabolites.

While our gas chromatographic-mass spectrometric methods required that we decide in advance which compounds to quantitate, no such requirement was imposed by the high performance liquid chromatographic procedures employing radiolabelled benzene. These techniques allowed us to demonstrate the metabolism of benzene by bone marrow in situ and to study the in vitro metabolism of benzene, including conjugate formation.

As this publication is intended to be a description of methods available for the study of xenobiotic disposition, only brief examples of the data obtained

---

**Table 2. Concentrations of phenol, catechol, and hydroquinone in venous blood of isolated hind limb perfusion after injection of benzene into bone marrow in situ.**

| Collection period, min | Phenol, pmole/10 min \(a\) | Catechol, pmole/10 min \(a\) | Hydroquinone, pmole/10 min \(a\) |
|------------------------|-----------------------------|-----------------------------|-------------------------------|
| 0-10                   | 4540 ± 773                  | 222 ± 74                    | 93 ± 7                        |
| 10-20                  | 664 ± 151                   | 59 ± 22                     | 11 ± 5                        |
| 20-30                  | 247 ± 41                    | 184 ± 117                   | 4 ± 3                         |
| 30-40                  | 291 ± 29                    | 49 ± 20                     | 11 ± 8                        |
| 40-50                  | 192 ± 123                   | 68 ± 27                     | 12 ± 10                       |
| 50-60                  | 58 ± 28                     | 21 ± 9                      | 11 ± 7                        |

\(a\)Values are means for 3-4 preparations ± S.E.M.
have been given. A fuller presentation and discussion of the disposition data can be found in the cited publications.

REFERENCES

1. Rickert, D. E., Baker, T. S., Bus, J. S., Barrow, C. S., and Irons, R. D. Benzene disposition in the rat after exposure by inhalation. Toxicol. Appl. Pharmacol. 49: 417 (1979).

2. Irons, R. D., Dent, J. G., Baker, T. S., and Rickert, D. E. Benzene is metabolized and covalently bound in bone marrow in vivo. Chem-Biol. Interact. 20: 241 (1980).

3. Greenlee, W. F., Rickert, D. E., Chism, J. P., and Irons, R. D. Effect of pretreatment with Aroclor 1254 and polychlorinated biphenyl isomers on the toxicity and metabolism of benzene. Abstracts, 19th Annual Meeting, Society of Toxicology, p. A45, 1980.

4. Synder, R., and Kocis, J. J. Current concepts of chronic benzene toxicity. CRC Crit. Rev. Toxicol. 3: 265 (1975).