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EXCRETION, METABOLISM AND TISSUE DISTRIBUTION OF A SPIN TRAPPING AGENT, α-PHENYL-N-*TERT*-BUTYL-NITRONE (PBN) IN RATS

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The objective of this study is using radiolabelled PBN to determine the tissue distribution, excretion, and metabolism of PBN in rats in order to evaluate the effective time to trap free radical in appropriate tissue(s). Our results demonstrated that PBN is rapidly absorbed when it is injected intraperitoneally in the animal. PBN can be used as an effective spin trapping agent for a variety of tissues since it is evenly distributed among a wide range of tissues measured. Since there is no difference in the tissue concentrations and distribution pattern of PBN at 15, 30 and 60 min after injection of PBN, it is appropriate to choose any of these time intervals to terminate the experiment and extract the spin adduct. The excretion of PBN, however, is slow. The majority of the radioactivity (70%) was excreted by the first 3 days. Only 5.7% of radioactivity was collected from 3 to 14 days. The remaining 25% of the radioactivity may be in the form of expired $^{14}CO_2$. Trace amounts of radioactivity were recovered in the feces. PBN has probably only one major form of metabolite excreted in the urine. A small amount of the parent compound, however, was also excreted in the urine. The chemical structure of the metabolite(s) is still unknown.

KEY WORDS: α-Phenyl-N-*tert*-butyl-nitrone (PBN), ^{14}C-PBN, PBN-metabolite, urinary excretion of PBN, tissue concentration of PBN, tissue distribution of PBN, and metabolism of PBN.

INTRODUCTION

α-Phenyl-N-*tert*-butyl-nitrone (PBN), a diamagnetic compound, is one of the most widely used spin trapping agents to detect unstable free radicals in both *in vitro* or *in vivo* biological systems.[1-6] In the *in vitro* biological system, PBN is often directly added to the microsomal or cell culture incubation medium to trap the free radicals generated from metabolism of toxins.[4,7,8] Although PBN has been shown to affect the mixed function oxidases (MFO) in hepatocytes,[7] the amount of PBN which can be metabolized by the microsomes of cell culture system is probably limited. The trapping efficiency of PBN is shown to be adequate. However, to detect free radical generation from toxins in the intact animals, PBN is often injected intraperitoneally immediately before or after the administration of toxin. Although PBN has been shown to be able to trap free radicals of CCl_4 in rats[4,5] and 3-methylindole in goats,[8] the tissue distribution, metabolism and toxicity of the spin trap, PBN, itself have not been documented. It is possible that there is an extensive metabolism of PBN in various tissues. Furthermore, the appropriate time for the measurment of concentrations of

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PBN-spin adduct after administration of the PBN and the toxin to the animal is often an educated guess.

The objectives of this study is using radiolabelled PBN to determine the tissue distribution, excretion, and metabolism of PBN in rats.

MATERIALS AND METHODS

PBN was obtained from Sigma (St. Louis, MO). α-Phenyl-α-[^{14}C] N-*tert*-butyl nitrone was custom synthesized by Amersham (Arlington Heights, IL). The specific activity of ^{14}C-PBN was 2.7 mCi/mmol with radiopurity of 98.15%. Methanol (HPLC grade) was obtained from Fisher Scientific (Toronto, Ontartio). Tissue solubilizer, Protosol, and scintillation counting fluid, Atomlight, were purchased from New England Nuclear (Boston, MA).

Male Wistar rats (Charles River, Montreal, Quebec) weighing 200–250 g were housed individually in suspended stainless stell metabolic cages in a temperature- and humidity-controlled room with light from 0800 to 2000 h. Rats were fed ad libitum with a chow diet (Purina, St. Louis, MO) and with free access to water.

To study the excretion rate of PBN, ^{14}C-PBN at a dose of 75 mg containing 1 μCi/kg body weight in saline solution was injected intraperitoneally in rats. Urine was collected and the volumes were recorded at a frequent intervals for the first 3 days (1, 4, 8, 12, 24, 48 and 72 h), and the residual radioactivity in urine was continuously monitored for 2 weeks after PBN administration. Urine samples of 1.0 ml from each time interval were taken for the determination of radioactivity in 10 ml of Atomlight by liquid scintillation spectrophotometry (Beckman LS 3801). The accumulative excretion of radioactivity in the urine was calculated and expressed as percentage of the dose. Urine collected at each time interval was analyzed for its PBN content and any radioactive metabolites using a reverse-phase liquid chromatographic system.

Methanol (3.5 ml) were added to 1.5 ml of urine samples. The mixture was vortexed and centrifuged at 2000 × g for 15 min. The supernatant was ready for HPLC injection after filtration through the Acrodisc Millipore, Bedford, MA). The HPLC was equipped with a dual piston pump (Varian 2010), a variable UV absorbance detector (Varian 2050) set at 289 nm, and a μ Bondapac™ C_{18} column (4.6 mm × 25 cm with 5–6 μm packing, Waters Division of Millipore). The mobile phase was a mixture of 70% methanol in water (v/v). The flow rate was 1.0 ml/min, and the spectrum was recorded by a printer (Linear 1200) at a chart speed of 1.0 cm/min.

The feces were collected and weighed daily. The radioactivity in the feces was determined by extracting 1 g of dried ground-up feces 4 times with initial 10 ml, following 5 ml of methanol. The methanol extracts were combined and then made up to a total of 25 ml. The radioactivity in 5 ml of the extract was determined in the scintillation fluid.

To study the tissue distribution, the animals were sacrificed at 15 and 30 min after intraperitoneally injection of PBN since the majority of the radioactivity was excreted after 1 h. Blood samples were withdrawn immediately from the left ventricle with heparinized tubes. Various tissues were removed and the weights were recorded. Aliquots (100 mg) of liver, heart, lung, spleen kidney, brain, prostate and seminal vesicle, muscle, adipose tissue, plasma and urine were solubilized in 1 ml of protosol. The radioactivity were determined in 10 ml of scintillation solution, Atomlight by liquid scintillation spectrophotometry (Beckman LS 3801).

Tissue samples (1 gm), along with 0.1 ml of 0.01 M phenacetin solution in ethanol, were homongenized in 2 ml of 0.15 M phosphate buffer, pH 7.4. The phenacetin solution was used as the internal standard for calculation of recovery. Unmetabolized PBN was extracted from the tissues with 15 ml of chloroform followed by three consecutive extractions with 10 ml of chloroform. The combined chloroform extracts were centrifuged at 4,400 × g for 10 min to ensure a clear solution and evaporated under vacuum to dryness. The extract was dissolved in 5.0 ml of 70% methanol/water and then filtered through Acrodisc. Since PBN is light sensitive, all glassware used throughout the extraction procedures were protected from light.

RESULTS AND DISCUSSION

The urinary excretion pattern of radioactivity after the injection of ^{14}C-PBN is shown in Figure 1. The clearance rate for ^{14}C-PBN in urine was relatively slow. Only 5% of radioactivity was excreted by 1 h and 50% by 12 h. The majority of the radioactivity (70%) was excreted by the first 3 days. Only 5.7% of radioactivity was collected from 3 to 14 days. Total radioactivity recovered from urine was 75.7% of the dose by 14 days. There was approximately 25% of radioactivity which could not be accounted for from the total urinary excretion. Judging from the position of the ^{14}C-label and the chemical properties of ^{14}C-PBN, it is most likely that the remaining 25% of radioactivity is either expired as $^{14}CO_2$ or deposited in the adipose tissue. Only 1–2% was recovered from the feces by day 3. Trace amounts of radioactivity in the feces were observed from day 3 to day 14. The feces were contaminated with spilled feeds, thus the total amount of radioactivity in feces was difficult to obtain accurately.

There were 4 major peaks (A, B and C + D) and 1 minor peak (E) observed by U.V. detector (289 nm) in the HPLC chromatogram of urine samples from 1 to 24 h (Figure 2). However, when the radioactivity in the HPLC eluant was determined, only peaks A and C + D exhibited radioactivity. (Using the current HPLC system, peak D is inseparable from peak C; we abbreviate these 2 peaks as C + D). The total radioactivity of peaks A and C + D accounts for 100% of the radioactivity injected into the HPLC. Peaks B and E contained no radioactivity. Peak A (retention time = 4.7) was confirmed to be the unmetabolized ^{14}C-PBN, therefore we assume

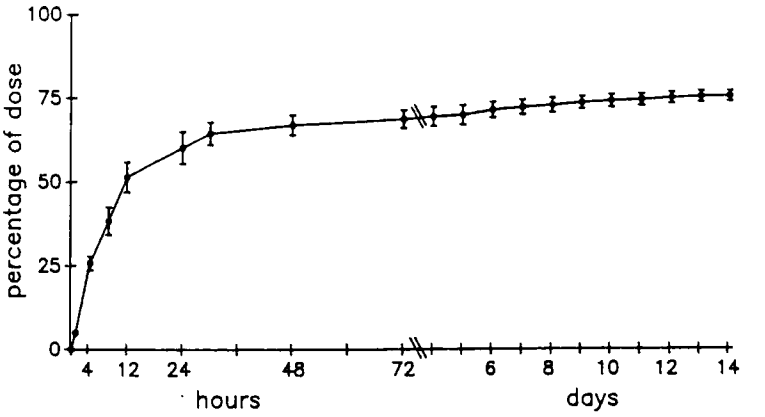

FIGURE 1 Accumulated radioactivity as percent of the dose excreted in the urine

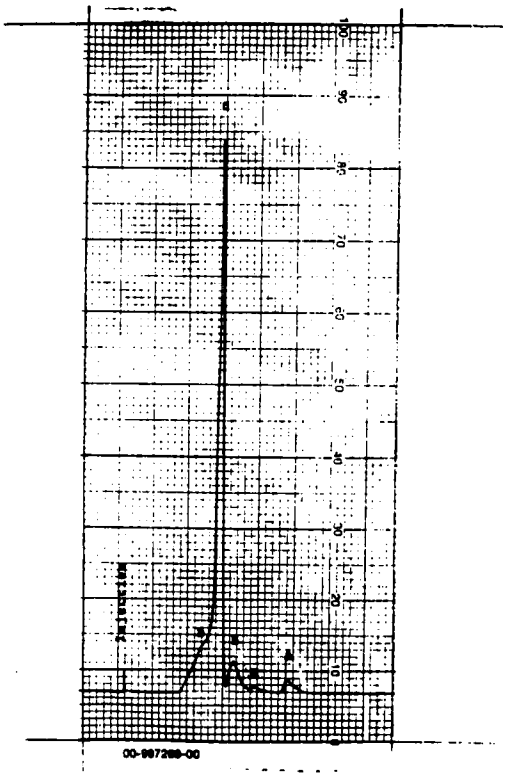

FIGURE 2 HPLC chromatogram of PBN and its metabolite in the urine as detected by U.V. absorbance at 289 nm.

that peak C + D (retention time = 2.8) contains the major metabolites. Since only the α-carbon of the PBN molecule was labelled with ^{14}C, any metabolite(s) of PBN without α-carbon can not be detected by the scintillation spectrophotometric method. Peak C + D can be detected by both radioactivity and U.V. at 289 nm, thus the structures of the metabolites in peak C + D contain both the α-carbon and the phenyl ring. We have eliminated the possibilities that benzaldehyde, p-hydroxybenzaldehyde, 2-hydroxybenzaldehyde, phenol, p-hydroxybenzoic acid and 2-hyroxybenzoic acid are the metabolites in peak C + D. It is possible that the metabolite(s) in

TABLE 1
The percent of PBN (Peak A) and its metabolites (Peak C+D) in rat urine excreted at different times

Time	PBN (Peak A)		Metabolites (Peak C + D)	
	dpm	%	dpm	%
1 hr	76.6 ± 12.6	13.3	566 ± 34	86.5
4 hr	85.2 ± 8.0	4.2	1986 ± 142	95.8
8 hr	50.8 ± 7.4	4.1	1490 ± 328	96.0
12 hr	35.7 ± 6.3	2.0	1720 ± 368	98.0
24 hr	12.1 ± 5.5	1.3	1058 ± 98.6	98.7

Mean ± SE, 4 animal in each group

TABLE 2
Recovery of radioactivity in tissues at different time intervals after injection of ^{14}C-PBN

Tissues	15 min	30 min (dpm/gm of fresh tissue)	60 min
Liver	2847.0 ± 170.9	3030.0 ± 102.1	2759.0 ± 205.2
Heart	3974.7 ± 1482.3	2351.4 ± 159.5	1965.4 ± 98.4
Lung	2306.9 ± 142.4	2204.7 ± 132.5	1883.5 ± 44.5
Spleen	2010.8 ± 68.6	1583.9 ± 169.6	1490.5 ± 39.5
Kidney	3284.3 ± 95.8	2952.8 ± 165.5	2884.7 ± 105.0
Brain	2848.4 ± 526.6	2295.2 ± 125.8	1971.9 ± 151.1
P & SV	2788.2 ± 65.0	2715.8 ± 200.5	2132.8 ± 173.8
Muscle	2358.1 ± 102.7	2229.5 ± 188.3	1708.8 ± 87.8
Fat	5680.9 ± 1494.6	5141.5 ± 1145.3	4887.4 ± 254.7
Plasma	2409.5 ± 72.2	2275.7 ± 100.1	2014.2 ± 135.0

Mean value ± SE; three animals in each group

peak C + D is a hydroxylated ^{14}C-PBN and/or its derivative. The chemical structures of peak C + D are still under investigation.

Table 1 shows the distribution and recovery of radioactivity between peak A and C + D. The majority of the radioactivity excreted in the urine was the PBN metabolites (C + D). However, the parent compound, PBN is also excreted in the urine. It was difficult to detect PBN in the urine after 12 h, since 98% of radioactivity was found to be the PBN-metabolites.

The tissue concentrations of radioactivity at 15, 30 and 60 min after injection of ^{14}C-PBN are shown in Table 2. Except for adipose tissue, ^{14}C-PBN was evenly distributed among all tissues measured. It seems that ^{14}C-PBN is capable of passing through the blood-brain barrier since the brain tissue and plasma have similar concentrations of ^{14}C-PBN. There is no difference in the pattern of tissue concentration of radioactivity measured at 15, 30 and 60 min. At 1 hour, when calculating the total radioactivity from all tissues measured as percent of the dose, the recovery was 94.1%, which is exactly the difference between the dose injected and the amount excreted in the urine.

The total radioactivity of ^{14}C-PBN calculated as percent of dose for each tissue at 15, 30 and 60 min after injection of ^{14}C-PBN is illustrated in Table 3. Of the ^{14}C-PBN injected, 52–58% was recovered from the 7 tissues measured. The remaining 42–48% of the radioactivity was distributed among plasma, muscle, adipose tissue, urine and

TABLE 3
Total radioactivity of ^{14}C-PBN recovered from each tissue as percent of dose injected

Tissue	15 min	30 min (% of ^{14}C-PBN)	60 min
Liver	34.51 ± 2.60	35.01 ± 2.53	31.53 ± 1.85
Heart	2.63 ± 0.12	2.64 ± 0.15	2.03 ± 0.09
Lung	3.49 ± 0.39	3.12 ± 0.28	2.96 ± 0.06
Spleen	1.77 ± 0.01	1.66 ± 0.17	1.62 ± 0.35
Kidney	9.88 ± 0.37	8.46 ± 0.41	8.51 ± 0.32
Brain	3.30 ± 0.31	3.10 ± 0.33	2.75 ± 0.44
P & SV	2.59 ± 0.21	3.10 ± 0.33	2.75 ± 0.44
Total	58.3	57.1	52.1

Mean value ± SE; 3 animals in each group

TABLE 4
Concentrations of PBN in tissues at different times following administration

Tissue	15 min	30 min	60 min
		(μg/gm of fresh tissue)	
Liver	56.39 ± 5.02	68.63 ± 2.03	52.88 ± 2.35
Kidney	45.25 ± 4.72	64.02 ± 4.18	44.68 ± 7.47
Heart	37.27 ± 5.13	52.31 ± 0.75	36.58 ± 5.28
Lung	41.98 ± 4.74	46.28 ± 1.18	37.21 ± 5.21
Brain	39.17 ± 4.46	50.64 ± 1.99	37.68 ± 5.87

Mean ± SEM. 4 animals in each group

others. Liver alone, is accountable for 30–35% of total radioactivity. Although the specific activity is the highest in adipose tissue (see Table 2), liver probably contains the highest total ^{14}C-PBN. It is possible that liver is the site of metabolism and adipose tissue is the site of storage for PBN.

When unlabelled PBN was injected (i.p.) and the unmetabolized PBN in 5 major tissues was extracted, the pattern of distribution of PBN in these tissues (Table 4) agrees with the results obtained when radiolabelled PBN (Table 2) was used. The unmetabolized PBN is evenly distributed among these selected tissues, since there were no differences ($p > 0.05$) in the concentration of the unmetabolized PBN. However, the PBN-metabolite in these tissues was not determined.

Figure 3 illustrates the rapid absorption in the blood of the intraperitoneally injected unlabelled PBN. Within 15 min, the plasma concentration of PBN reached a plateau. However, statistically there is no difference in the plasma concentrations of PBN measured before 1 h.

The results demonstrate that PBN is rapidly absorbed when it is injected intraperitoneally in the animal. PBN can be used as an effective spin trapping agent for a variety of tissues since it is evenly distributed among a wide range of tissues measured. Since there is no difference in the tissue concentration or distribution pattern of PBN at 15, 30 and 60 min after its injection, it is appropriate to choose any of these time intervals to terminate the experiment and extract any spin adducts that may be present. The excretion of PBN, however, is slow. PBN has probably only one

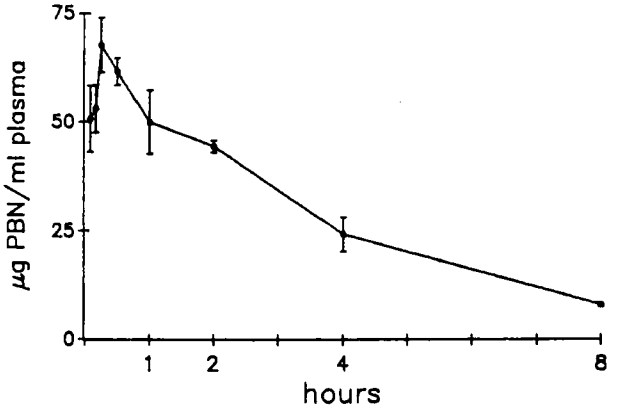

FIGURE 3 Plasma concentration of unmetabolized PBN after i.p. injection of non-radioactive PBN.

major metabolite excreted in the urine. A small amount of the parent compound, however, is also excreted in the urine. The identity of the metabolite(s) is still unknown. Until the remaining 25% of the radioactivity can be accounted for, PBN may not be a safe agent to use in any human experiments.

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