Distribution and Metabolism of 1-Propyl-1-nitrosourea in Rats

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ABSTRACT

The carcinogen 1-[14C]propyl-1-nitrosourea (PNU) was readily absorbed from the rat gut, and the radioactivity was excreted mainly in the urine and expired air. The urinary metabolites of PNU were 1-propylurea and urea. 1-Propylurea was shown to be excreted largely unchanged in the urine. Both [14C]PNU and 1-[14C]propylurea were found to be eliminated rapidly from the rat body. Besides CO2 from PNU, isopropyl alcohol was identified as a volatile metabolite in the expired air.

Specific high concentrations (%/g) in main organs and tissues were not observed in adult rats 24 hr after single p.o. doses (20 mg/kg) of labeled PNU. The ureido carbon of PNU showed considerable retention in the blood, while relatively high residual levels were found in the liver for the propyl carbon. Autoradiographic studies on pregnant rats showed a uniform distribution between maternal and fetal bodies a short time after dosing. A relatively high concentration of 14C label was found in the maternal blood 24 hr after treatment with [carbonyl-14C]PNU. Localization of radioactivity in bone systems such as the fetal sterna and vertebræ was noted 6 hr after treatment with [propyl-1-14C]PNU. Metabolic pathways of PNU in the rat are proposed.

INTRODUCTION

In 1972, Druckrey (3) reported on the incidence of duodenal tumors in the rat with PNU, and Ivanovic and Zeller (8) induced neurogenic tumors and nephroblastoma in the offspring of pregnant BD II rats by administration of PNU. Recently, Ogiu et al. (13) found that leukemia and digestive tract tumors developed at an incidence of 57 and 28%, respectively, in female Donryu rats continuously given PNU in spring of pregnant BD II rats by administration of PNU.

The metabolism of some /N-nitrosoureas such as methyl (10, 17), ethyl (9, 18), and butyl (7) derivatives has already been studied in the rat or mouse using the tracer technique. How ever, detailed studies on the metabolism of PNU in rats have not been reported in the literature. This fact prompted us to investigate the metabolism of PNU in female Donryu rats.

MATERIALS AND METHODS

Chemicals. PNU and PU were synthesized in our laboratory. PNU showed m.p. 77.0–78.0°C (4) and UV max 236 nm (log ε 3.82). PU melted at 103.0–104.5°C (21). Urease (EC 3.5.1.5) type II, 2800 units/g, was obtained from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals and solvents were reagent grade.

Starting materials, [propyl-1-14C]propyl amine-HCl (1.99 mCi/mmol) and potassium [14C]cyanate (57 mCi/mmol), were purchased from California Bionuclear Corp. (Sun Valley, Calif.) and the Radiochemical Centre (Amersham, England), respectively. [propyl-1-14C]PNU and [carbonyl-14C]PNU were synthesized in about 30% yield on a mmol scale by a method reported previously (4). [propyl-1-14C]PNU and [carbonyl-14C]PU were obtained as intermediates during [14C]PNU synthesis. Labeled PNU exhibited a single spot on TLC, and its radiochemical purity was above 99%. The specific radioactivities of the labeled chemicals used are shown in the appropriate tables.

Animals and Treatment. Female Donryu rats weighing from 200 to 245 g were used after fasting overnight prior to p.o. administration of labeled PNU or PU in a dose of 20 mg/kg. A dose of PNU (200 mg/kg) was used to study organic metabolites in expired gases from the rat.

The autoradiographic study in pregnant rats was done on Day 19 of gestation according to the experimental conditions of Ivanovic and Zeller (8). Rats weighing from 270 to 300 g were given 4.7 to 6.5 μCi of [14C]PNU p.o. in a dose of 200 μCi/kg. Rats were sacrificed 1, 3, 6, and 24 hr after dosing.

Whole-Body Autoradiography. All of the rats were processed for whole-body autoradiography by the Ullberg technique. Whole sagittal sections, 20 μm thick, were applied to RI-70 Scotch tape (Hisamitsu Pharmaceutical Co., Fukuoka, Japan). The freeze-dried sections were placed in contact with Sakura X-ray film (Konishiroku Co., Tokyo, Japan) for 3 months to prepare the autoradiograms.

Instruments. A gas chromatograph (Shimadzu 5APF) equipped with a single-ionization detectors was used for estimation of biodegraded products of PNU in the expired gases of the rat. The 2-m x 3-mm inside diameter gas column was packed with 5% diethylene glycol succinate on 60 to 80 mesh Chromosorb W. Trapped-breath volatiles were analyzed on a DuPont Dimaspec GC-MS Model 321 connected with a Model 320 data system at 70 eV. The GC column used with the integrated GC-MS instrument was 1.5-m x 4-mm inside diameter glass tube packed with 10% Theron-1500 on Chromosorb W, acid-washed dimethylchlorosilane 80 to 100 mesh. Operating conditions were as follows: column temperature, 45°; and helium flow rate, 40 ml/min.

PC and TLC. Paper sheets (Toyoroshi No. 51A, 2 x 40 cm, and Whatman No. 3MM, 46 x 57 cm) and silica gel spot film containing a fluorescent indicator were used for analyses of PNU metabolites. Two solvent systems were used for PC: Solvent A, n-propyl alcohol:n-butyl alcohol:water (2:3:2); and Solvent B, n-butyl alcohol:acetic acid:water (4:1:2). Three solvent systems were used for TLC: Solvent C, aceto nitric acid:methanol:petroleum ether (b.p. 30–60°) (5:10:3); Solvent D, chloroform:ethanol (3:1); and Solvent E, dichloromethane:ethanol (1:1). PNU was visualized under UV light on the silica gel chromatogram, and areas were detected by Ehrlich reagent.

Radioassay Methods. Radioactivity was estimated on an LSC-651 scintillation counter (Aloka). A radioscanner (TRM-1B; Aloka) was used for scanning chromatograms. Urine and feces samples were assayed for 14C by liquid scintillation spectrometry as reported previously (20).
A portion of the tissue sample was dissolved in 1 ml of Soluene 350 (Packard) and then mixed with a toluene-based scintillator for counting.

**Processing of Urine and Feces.** The urine sample was lyophilized, and the residue was treated with acetone or tetrahydrofuran. The extract was concentrated, and an aliquot of the concentrated extract was subjected to PC. The developed chromatogram was cut into 1.5-cm strips, and the radioactivity on each strip was determined. Chromatograms containing lower radioactivities were radioautographed with authentic standards for comparison.

To confirm the identity of \([14C]PNU\) or \([14C]\)urea, reversed isotope dilution analysis was used. One volume of the urine sample was mixed with 6 volumes of 0.1 M Tris-maleate buffer (pH 7.01) in the presence of urease for identification of \([14C]\)urea. The mixture was allowed to stand for 2 days at room temperature with occasional shaking. The released \(^{14}CO_2\) was trapped in ethanolamine and counted (20).

The 24-hr fecal sample was extracted with methanol, and the concentrated extract was examined by TLC and PC to determine metabolites.

**Working Up of Blood and Stomach Contents.** The blood samples from 2 rats were obtained by cardiac puncture 1.5 hr after p.o. dosing with \([14C]PNNU\). The plasma and cell fractions were separated. A portion of plasma was counted directly in a dioxane scintillator, and an aliquot of cells was oxidized to \(^{14}CO_2\) for counting.

Stomach contents were extracted with acetone 1 hr after a p.o. dose of \([14C]PNNU\). The concentrated extract was subjected to TLC for metabolite analysis.

**Trapping and Confirmation of Volatile Metabolites.** Immediately after administration of \([14C]PNU\) or \([14C]\)PNU, each rat was placed in a separate glass metabolic chamber (Natsume Co., Tokyo, Japan). Air was drawn through the chamber at about 300 to 400 ml/min and then through 2 silica gel traps connected in series to trap volatile organic compounds and an ethanolamine trap to collect respiratory \(^{14}CO_2\). The silica gel trap contained 18 g active silica gel (6 to 10 mesh, medium granular blue; Ishizu Pharmaceutical Co.) in a U-type glass. Traps were changed every 24 hr. Volatile compounds on silica gel were eluted with 200 ml dichloromethane followed by 2 x 200 ml methanol and 100 ml dimethyl sulfoxide. The final eluate did not show significant radioactivity.

In the case of rats treated with unlabeled PNU, another metabolic chamber (Metabolica; Sugiyamagen Co., Tokyo, Japan) was used. Traps 1 and 2 each consisted of 10 ml ether in a glass test tube (1.6 cm in diameter and 19 cm long) immersed in a dry ice:acetone bath. A glass tube (1.2 cm in diameter and 10 cm long) cooled with a mixture of ice and salt was used to condense breath water before Traps 1 and 2. An aliquot of the ether traps was subjected to GC and GC-MS.

Silica gel traps similar to the tracer technique were also used in addition to ether traps. The silica gel was extracted with ether, and an aliquot of the ether extracts was analyzed by GC-MS.

**RESULTS**

**Stability of PNU in an Aqueous Solution.** The stability of PNU (4.3 mm) at various pH values was examined at 37° in 0.1 M Michael’s buffer (tartrate, pH 1.6:acetate, pH 3.70 to 5.0:phosphate, pH 5.20 to 7.0:ammonia/ammonium chloride, pH 8.50). Absorbance decay was monitored at 398 nm. Approximate half-lives were as follows: 20 hr (pH 1.60); 60 hr (pH 3.70); 140 hr (pH 4.10); 16 hr (pH 5.31); 7 hr (pH 6.02); 2 hr (pH 6.30); 30 min (pH 6.80); and 3 min (pH 8.50).

**Excretion of Radioactivity.** The excretion of radioactivity after administration of \([14C]PNNU\) and \([14C]PNU\) to rats is summarized in Table 1. The radioactivity was rapidly eliminated from the body in all cases. The main excretion routes of PNU appear to be the kidney and lung. About 18% of the radioactivity from the propyl group of PNU was recovered in expired gases other than \(^{14}CO_2\), while in the case of [carbonyl-\(^{14}C\)]PNNU expired radioactivity in forms other than \(^{14}CO_2\) was negligible.

Fecal elimination was a minor source of the administered radioactivity for both PNU and PU. The excretion rate of \(^{14}CO_2\) from the ureido carbon of PNU was almost constant for 3 rats, but marked variation in the production of \(^{14}CO_2\) and radioactivity remaining in the carcass was observed after [propyl-1-\(^{14}C\)]PNNU administration. Respiratory \(^{14}CO_2\) within the first 24 hr was 80 to 90% of the 2-day output, with saturation of the excretion rate at 6 hr after administration of \([14C]PNNU\).

Most \(^{14}C\) label from PU was excreted in the urine. An extremely low yield of \(^{14}CO_2\) suggested that PU remained almost intact in vivo. Radioactivity from labeled PNU and PU was quantitatively recovered in all cases.

**Tissue Distribution.** The distribution of total radioactivity 24 hr after administration of \([14C]PNNU\) to the rat is shown in Table 2. Target tissues such as the thymus and bone marrow that are closely related to the induction of leukemia were particularly scrutinized, but there was no evidence of a specific affinity for these tissues.

In the case of [propyl-1-\(^{14}C\)]PNNU, the maximum concentration (%/g) of the \(^{14}C\) label was seen in the liver, and bone marrow showed the second highest concentration, although the exact binding form in vivo is not clear at present. There was about a 1.6-fold greater retention of \(^{14}C\) label in the liver with [propyl-1-\(^{14}C\)]PNNU than with [carbonyl-\(^{14}C\)]PNNU. The \(^{14}C\) concentration in the blood was highest when the latter compound was administered and showed about a 5-fold greater retention than did [propyl-1-\(^{14}C\)]PNNU.

It may be assumed from the distribution data in the muscle that \(^{14}C\) label is distributed uniformly in the rat body irrespective of the \(^{14}C\) labeling position of the PNU molecule. The lowest

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**Table 1**

<table>
<thead>
<tr>
<th>Labeled compound</th>
<th>No. of rats</th>
<th>Breath volatile</th>
<th>CO(_2)</th>
<th>Urine</th>
<th>Feces</th>
<th>Carcass</th>
</tr>
</thead>
<tbody>
<tr>
<td>([propyl-1-^{14}C]PNNU (6.7 \muCi/mmol))</td>
<td>3</td>
<td>7.9 ± 5.2(^\text{a})</td>
<td>41.0 ± 16.6</td>
<td>24.3 ± 4.1</td>
<td>4.3 ± 1.7</td>
<td>11.6 ± 14.5</td>
</tr>
<tr>
<td>([carbonyl-^{14}C]PNNU (11.9 \muCi/mmol))</td>
<td>3</td>
<td>Neg.(^\text{b})</td>
<td>70.8 ± 8.1</td>
<td>23.5 ± 1.7</td>
<td>1.6 ± 0.4</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>([propyl-1-^{14}C]PNU (11.9 \muCi/mmol))</td>
<td>1</td>
<td>1.1(^\text{c})</td>
<td>2.5</td>
<td>93.8</td>
<td>1.1</td>
<td>1.0(^\text{d})</td>
</tr>
<tr>
<td>([carbonyl-^{14}C]PNU (8.1 \muCi/mmol))</td>
<td>2</td>
<td>Neg.</td>
<td>1.2(^\text{e})</td>
<td>91.5(^\text{f})</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^\text{a}\) Mean ± S.D. for 3 rats.  
\(^\text{b}\) Neg., negligible (<0.03%).  
\(^\text{c}\) Mean for 2 rats.
distribution and metabolism of PNU in rats

Table 2

<table>
<thead>
<tr>
<th>Organ and tissue</th>
<th>[propyl-1-14C]PNU (%/g × 100)</th>
<th>[carbonyl-1-14C]PNU (%/g × 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>2.1 ± 0.2</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td>Heart</td>
<td>2.1 ± 0.3</td>
<td>8.6 ± 2.0</td>
</tr>
<tr>
<td>Lung</td>
<td>4.2 ± 0.5</td>
<td>6.8 ± 1.4</td>
</tr>
<tr>
<td>Liver</td>
<td>12.4 ± 0.3</td>
<td>7.6 ± 1.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.4 ± 0.9</td>
<td>4.2 ± 3.5</td>
</tr>
<tr>
<td>Intestine</td>
<td>5.6 ± 3.0</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>5.5 ± 0.5</td>
<td>9.2 ± 1.9</td>
</tr>
<tr>
<td>Stomach</td>
<td>6.5 ± 0.9</td>
<td>5.3 ± 1.1</td>
</tr>
<tr>
<td>Ovary</td>
<td>6.4 ± 2.4</td>
<td>5.8 ± 2.2</td>
</tr>
<tr>
<td>Uterus</td>
<td>4.9 ± 0.9</td>
<td>7.5 ± 1.8</td>
</tr>
<tr>
<td>Thymus</td>
<td>4.9 ± 0.3</td>
<td>5.2 ± 1.1</td>
</tr>
<tr>
<td>Adrenal</td>
<td>7.7 ± 0.7</td>
<td>6.9 ± 1.4</td>
</tr>
<tr>
<td>Muscle</td>
<td>5.0 ± 3.0</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td>Blood</td>
<td>3.1 ± 0.6</td>
<td>15.1 ± 6.3</td>
</tr>
<tr>
<td>Adipose</td>
<td>0.6 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>7.9 ± 0.7</td>
<td>7.9 ± 0.9</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
* Assumed 40% of body weight.
* Assumed 7.69% of body weight.
* Assumed 14% of body weight.
* Assumed 4 g/rat.

Concentration was found in adipose tissue.

In a separate experiment on the distribution of radioactivity in the blood, radioactivity was found in both cellular and plasma fractions. The RBC activity:plasma activity ratios were 3.3 for [propyl-1-14C]PNU and 1.0 for [carbonyl-1-14C]PNU in 2 experiments.

 Autoradiography. Figs. 1 to 4 are prints of autoradiograms. With [carbonyl-1-14C]PNU, a considerable amount of radioactivity was found in the blood of the dam after 3 hr and still remained after 24 hr. This fact is consistent with the result of the distribution study described above.

On the other hand, when [propyl-1-14C]PNU was administered to the rat, the 14C concentration in the fetuses was approximately equivalent to that of the dam after 1 hr. Thereafter, at 3 and 6 hr, the radioactivity of the fetal liver was the same level as that of the maternal liver. At 6 hr, the radioactivity in the fetal vertebrae and sterna was higher than that at 3 hr except for maternal salivary glands. At 6 hr, the concentrations of the maternal blood, heart, and kidney were similar to that of the total liver except for low activity in the femur. Most radioactivity was eliminated after 24 hr. No blood-placenta barrier was observed for PNU and its metabolites in rats.

Identification of Metabolites. Identification of radioactive PU and urea in the urine was based on cochromatography with authentic standards. The Rs values of standards were as follows. Solvent A: PU, 0.83; urea, 0.35. Solvent B: PU, 0.85; urea, 0.47. Rs values of [14C]PNU metabolites were 0.84 and 0.36 for Solvent A and 0.85 and 0.47 for Solvent B. The Rs values of [14C]PU metabolites were 0.82 and 0.85 for Solvents A and B, respectively. Finally, 2 metabolites were confirmed by a reverse-isotope dilution method; i.e., the specific activity (dpm/mg) of PU from the following recrystallizations of acetone were: first, 2020; second, 2410; third, 2387; and fourth, 2388. The specific activities of urea for the following recrystallizations from ethanol were: first, 1579; second, 992; third, 997; and fourth, 1013.

PNU resulted in the production of PU as a main metabolite, but not degradation products formed by ω - or ω-1 oxidation of PU. PNU was not excreted in the urine upon administration of PNU because the spot of PNU with an Rs of 0.76 for Solvent D disappeared completely from the urine.

Furthermore, the radioactive zone of urea on Whatman No. 3MM paper was eluted with distilled water. When an aliquot of the eluates was treated with 45 mg of urease, about 30% of the added radioactivity was recovered as 14CO2 whereas the conversion of [carbonyl-1-14C]PU to 14CO2 was negligible. Thus, [14C]urea was isolated only in the case of [carbonyl-1-14C]PNU administration. In a similar way using Solvents A and E, PU alone was identified in the feces of rats given labeled PNU or PU.

TLC analysis of stomach contents revealed the presence of 2 radioactive materials with Rs values of 0.59 and 0.05 for Solvent C and 0.85 and 0.58 for Solvent E. The substance with higher Rs values corresponded to PNU, and the substance with lower Rs values was identical to PU.

The GC peak of volatile products under the conditions described in Chart 1 indicated that one of the volatile metabolites had a retention time (1.4 min) identical with that of isopropyl alcohol. By GC-MS, its mass spectrum gave a molecular ion at m/e 60 with prominent peaks at m/e 59(M — 1) and m/e 45 (M—CH3). The base peak at m/e 45 shows the presence of CH3—CH=0+H which is most noticeable in the spectra of secondary alcohols. This mass spectrum was identical with that of isopropyl alcohol. The peak at 6.1 min was not identical with that of acetone, and its identity remains to be determined.

The above findings demonstrate that the main metabolite in rat breath is isopropyl alcohol. No n-propyl alcohol was detected under the given experimental conditions.
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DISCUSSION

The stability of PNU in various buffer solutions was analogous to that of its homologs, MNU, ENU, and BNU, as reported previously (5–7). In general, it is accepted that 1-alkyl-1-nitrosoureas spontaneusly undergo rapid heterolytic decomposition, generating unstable moieties. Thus, as in vitro (15), under physiological conditions the formation of n-propyl alcohol and isopropyl alcohol would most probably occur in vivo via a pathway which produces propyl-diazohydroxide (or carboxylic acid, CH₃C₂H₅CH₂⁺ and CH₃CH₂CH₂⁺) (14). In this study, we demonstrated the presence of isopropyl alcohol but not n-propyl alcohol in the expired gases from the rat receiving PNU. The present result suggests different turnover rates of the n- and iso forms of propyl alcohol in vivo (1, 12).

Until now, there have been no detailed data on vaporizable organic metabolites of MNU, ENU, and BNU. By using silica gel traps, satisfactory recovery of radioactivity was obtained in this study. The reason for poor ¹⁴C recovery (70%) in [methyl-¹⁴C]MNU experiments (17) is most probably due to insufficient trapping of volatile metabolites other than CO₂. PNU is simply produced by denitrosation of PNU, whereas the formation of urea requires the recombination of formed isocyante or released CO₂ with ammonia in vivo. Thus, [¹⁴C]urea was detected only when [carbonyl-¹⁴C]PNU was given to the rat. On the basis of these results on the PNU metabolites, the proposed pathways of PNU metabolism in the rat are shown in Chart 2.

The autoradiograms in pregnant rats demonstrated rapid transplacental passage and ubiquitous distribution of radioactivity associated with [¹⁴C]PNU, indicating that PNU and its metabolites freely crossed the placenta. The localization of radioactivity in the bone systems such as fetal sterna and vertebrae is a very interesting finding, but its significance remains to be clarified.

We have reported on the binding of [¹⁴C]PNU with DNA, RNA, proteins, and synthetic biopolymers in vitro (11). Similar to the butyl group from BNU (7), binding of the propyl group from PNU was not observed to any detectable extent, except for albumin and polyhistidine, whereas the degree of carboxymylation of histone, albumin, polylysine, and polyhistidine by the ureido carbon of PNU was nearly the same as that for MNU and ENU. The extent of alkylation of nucleic acids decreased with an increase in the alkyl chain length of 1-alkyl-1-nitrosoureas.

In relation to the carcinogenicity of MNU and ENU, a great deal of useful information has been reported concerning the interactions of these compounds with DNA in vivo and the elimination of alkylated bases in DNA (16). However, only minimal data are available concerning the actions of PNU on DNA and RNA in vitro (2).

Further detailed studies on the metabolism of PNU in rats, particularly the reactions of PNU metabolites with cellular components in the hematopoietic organs, will be necessary to elucidate the leukemogenic mechanism of PNU.

ACKNOWLEDGMENTS

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REFERENCES


Figs. 1 to 4. Autoradiogram of pregnant Donryu rats after p.o. administration of [14C]PNU (200 mg/kg) on Day 19 of gestation. Figs. 1 and 2 are typical prints of whole-body autoradiogram following p.o. [carbonyl-14C]PNU administration. In Fig. 1, there is a high concentration in the blood (involving heart blood and lung blood). The activity in the fetuses and dam is equal to that of the blood. Fig. 2 shows that the activity in the blood is still very high after 24 hr. Some radioactivities are observed in the liver of dam and fetuses. No accumulation is seen in the maternal brain.
Figs. 3 and 4 are prints of whole-body autoradiograms after p.o. (propyl-1-\(^{14}\)C)PNU dosing. Fig. 3 shows relatively high concentration in the fetal and maternal livers, and the radioactivity in the femur (bone) of the dam remains higher than that of her kidney. Fig. 4 shows that the radioactivity in the fetal and maternal livers is still high after 6 hr. Some radioactivities are visible in the maternal salivary glands. The clear indication of fetal sterna and vertebrae is a unique finding.
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