

Physiological Disposition of 1-Acetyl-2-picolinoylhydrazine (NSC 68626) in Rats Bearing Walker Carcinoma 256¹

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SUMMARY

The purpose of the study was to investigate distribution and metabolism of 1-acetyl-2-picolinoylhydrazine in tissues of tumor-bearing rats in order to understand the basis of its toxicity and antitumor activity. Drug labeled with ¹⁴C in the carbonyl moiety was used to determine drug-derived radioactivity. A procedure involving chloroform extraction and paper chromatography was used to determine the unchanged drug. Peak levels of radioactivity were attained in all organs within 4 hr after a therapeutic i.p. dose and within 15 min after a low i.v. dose. Although no preferential uptake of 1-acetyl-2-picolinoylhydrazine was found in any organ, the uptake by brain was high and comparable to that seen in spleen and tumor and may account for the neurotoxicity and antitumor activity. Disappearance of 1-acetyl-2-picolinoylhydrazine from tissues was rapid; more than 90% was removed within 6 hr after the i.p. therapeutic dose administration. Metabolism of the drug was rapid regardless of the route or dose. More than 50% of the drug-derived label in plasma was found to be in the form of metabolites within 2 min after i.v. administration. Fifty-five % of the injected radioactivity was excreted in urine within 6 hr and 95% was excreted by the end of 24 hr; only trace amounts (2 to 4%) of the unchanged drug were found in urine at any time interval. Several metabolites were detected in urine by paper chromatography, and one of them appeared to be picolinic acid.

INTRODUCTION

APH³ has been shown to inhibit Walker carcinoma 256 (75 to 100%) when given by the p.o., s.c., i.m., or i.p. routes.⁴ Pharmacological studies in humans (6, 7) have shown that the drug, given i.v., caused neurotoxicity that was reversed by discontinuing the drug treatment. In view of the generally low toxicity of potentially effective tumor-inhibitory doses of the drug and its lack of hematological

toxicity (7) in particular, further study of its metabolism and distribution was deemed desirable.

MATERIALS AND METHODS

APH-carbonyl-¹⁴C was obtained from Monsanto Research Corporation, Dayton, Ohio, and purified by paper chromatography before use. APH and its analogs, picolinoylhydrazine (NSC 81875) and picolinamide (NSC 52473), were supplied by Drug Research and Development, National Cancer Institute, Bethesda, Md. Picolinic acid was purchased from Eastman Organic Chemicals, Rochester, N. Y. Soluene and Instagel were purchased from Packard Instrument Company, Downers Grove, Ill. Male rats (CR/RAR), 5 to 6 weeks old, weighing 50 to 60 g were used in all the experiments. Walker carcinoma 256 was implanted i.m. in the right leg of the animals.

Estimation of the Unchanged Drug in Biological Materials. A method based on solvent extraction and paper chromatography of the extract was developed to estimate unchanged drug in biological material. Tissues were homogenized in ice-cold distilled water (25% w/v). Urine and plasma were used without dilution. One-ml aliquots of the tissue homogenates, plasma, or urine were extracted 3 times with 5 ml of water-saturated chloroform. The chloroform extracts were pooled and evaporated to dryness. The residue was dissolved in 0.1 ml of an APH solution in water (0.5 mg/ml) and 40 μ l of the solution were spotted on Whatman No. 1 chromatographic paper. An ascending chromatogram was developed in butanol:water (50:8) for 17 hr. The chromatogram was dried in air, and the spots were visualized under UV. An area on the chromatogram corresponding to the pure drug was cut out and eluted in 2 ml of distilled water overnight in sealed test tubes. An aliquot (0.5 ml to 1.0 ml) of clear supernatant was counted in a Packard Model 3375 spectrometer. The counting efficiency was 82 to 84%.

Estimation of Total Drug. An aliquot (0.1 ml) of the tissue homogenates of plasma was dissolved in 0.4 ml of Soluene. To the clear solution, 10 ml of Instagel were added; the mixture was kept at 60° for 60 min and cooled to 4°, and its radioactivity was determined in the scintillation spectrometer. An aliquot of urine (0.1 ml) was mixed with 10 ml of Instagel and the radioactivity was determined in the same manner. The counting efficiency was 76 to 78%.

Specificity of the Procedure. Two rats were given 301.8 mg of APH (100 μ Ci) per kg i.p. and sacrificed after 1 hr.

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³ The abbreviations used are: APH, 1-acetyl-2-picolinoylhydrazine (NSC 68626); INH, isonicotinylhydrazine.

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Excised liver was homogenized and extracted with chloroform, and the extract was chromatographed as described above. A 1-inch-wide strip of the chromatogram was divided along its length into 1-inch pieces which were eluted and the radioactivity in the eluate was determined. The chloroform extracted only 14% of the total radioactivity present in the liver homogenate. The rest of the 86% radioactivity did not contain unchanged drug that could be detected by further extraction and paper chromatography. A radiochromatogram of the chloroform extract showed that most of the radioactivity (77%) came from the unchanged drug, indicating a fair degree of specificity of the drug in the extraction procedure. A routine paper chromatograph of the chloroform extract which separated the drug from its metabolites rendered the procedure still more specific for the unchanged drug. For determination of whether the procedure was efficient enough to extract most of the unchanged drug from biological material, 50 μ l of 2 mg/ml unlabeled APH solution were added to 1 ml of tissue homogenates (25% w/v) and extracted with chloroform. The extracts were evaporated to dryness. The residue was extracted with 4 ml of distilled water and the aqueous solution was centrifuged. The UV absorption of the clear supernatant was determined at 265 nm in a Gilford spectrophotometer. In a parallel experiment, tissue homogenates without the drug were analyzed. The percentage of recovery was calculated from the difference in absorbance between the extracts of tissues and the extracts of tissues containing the drug. The recovery of the drug was 93% from the aqueous solution and 89 to 92% from tissues, plasma, urine, and feces. The influence of pH on the extraction of the drug was also investigated. Aqueous drug solutions (100 μ g/ml) at various pH's were extracted with chloroform and evaporated to dryness. The residue was extracted with water cleared by centrifugation and assayed spectrophotometrically. The maximum extraction of the drug (93%) was achieved at pH 6.0 which remained unchanged until pH 8.0. Further increase in pH decreased the percentage of recovery of the drug. Since the pH's of urine, plasma, and tissue homogenates were within this range, no adjustment of pH was required before the extraction procedure.

Stability of APH at Neutral, Acid, and Alkaline pH. APH solutions (2 mg/ml) in water, 1 N HCl, and 1 N NaOH were incubated at 60°, and aliquots (0.1 ml) were removed after various time periods. The aliquots were neutralized with 1 N acid or base and 40 μ l of the mixture were analyzed by paper chromatography. Drug was found to be stable in aqueous solution for 24 hr but decomposed up to 80% in acid and 20% in alkali within 2 hr.

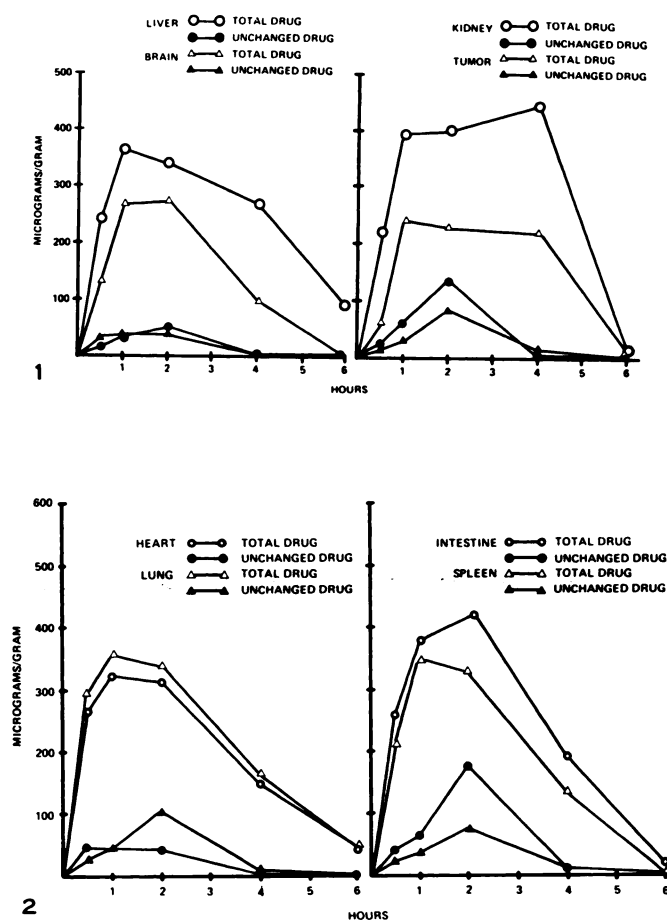
Tissue Distribution Studies. The unchanged drug and total drug equivalents (total radioactivity) in tissues and plasma were determined at various time intervals after the optimal therapeutic dose⁴ of 301.8 mg/kg (100 μ Ci) was given i.p. and a low dose, 1.75 mg/kg (79 μ Ci) i.v., were given to rats bearing an 8-day-old Walker tumor. Two rats were sacrificed at each time interval; organs were removed and homogenized in ice-cold water (25% w/v) for unchanged and total drug as described earlier.

Metabolic Studies. The 24-hr sample of urine from

drug-treated (APH, 301.8 mg/kg) animals was analyzed by 2-dimensional paper chromatography. An aliquot of urine (20 μ l) was spotted and an ascending paper chromatogram with butanol:water (50:8) was developed for 17 hr. The chromatogram was dried and developed a 2nd time with butanol:acetic acid:water (4:1:5) at right angles to the direction of the solvent flow used for the 1st time. The spots were visualized by UV, cut, and eluted in 2 ml of distilled water, and radioactivity in an aliquot (0.5 to 1.0 ml) of clear eluate was determined. Urine from untreated animals was analyzed in a similar way for reference. The compounds that could possibly be among the metabolic products of APH were also chromatographed and their R_F values in 2 solvent systems were determined.

RESULTS

Tissue Distribution. Unchanged drug and total radioactivity levels in various organs after administration of the therapeutic dose given i.p. are shown in Charts 1, 2, and 3. The drug concentration is expressed as μ g/g of tissue. The peak drug concentration was attained within 2 hr in all the organs except kidney (4 hr). The peak concentration of the



Charts 1 and 2. Levels of total and unchanged drug in tissues after i.p. administration. Tumor-bearing rats were given APH-¹⁴C, 301.8 mg (100 μ Ci)/kg i.p. and 2 rats were sacrificed at various time intervals.

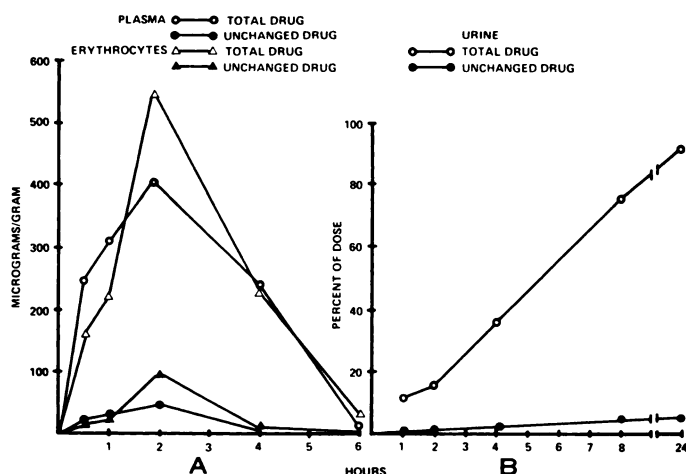
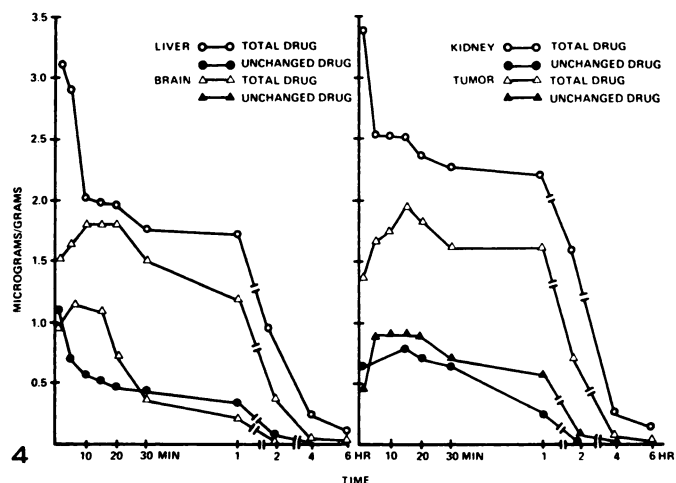


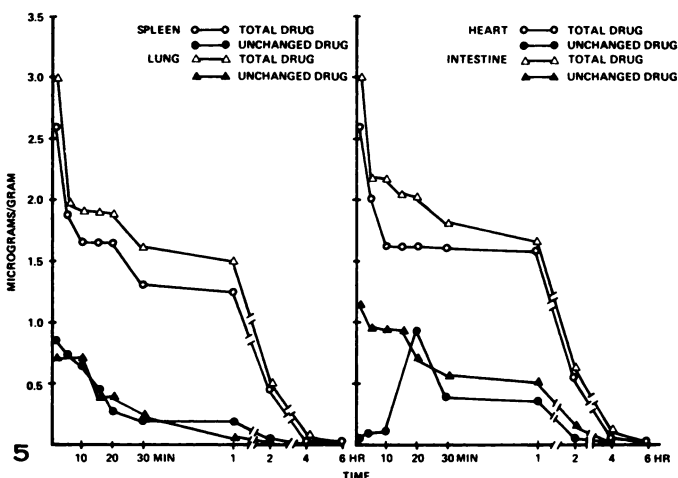
Chart 3. Levels of total and unchanged drug in plasma and erythrocytes and in urine. Tumor-bearing rats were given APH- ^{14}C , 301.8 mg (100 μCi)/kg i.p. and 2 rats were sacrificed at various time intervals to collect blood samples; another group of 2 rats were kept in a metabolic cage to collect urine.

total drug and the unchanged drug ranged from 270 to 450 and 40 to 180 $\mu\text{g/g}$ of tissues, respectively. The drug was extensively metabolized in tissues, and levels of the unchanged drug varied from 12 to 42% of the total radioactivity. Penetration of the drug-derived radioactivity into brain was high and comparable to that of liver and tumor, indicating that there was no specific blood-brain barrier for the drug. Disappearance of the drug from tissues was also rapid; more than 90% of the drug disappeared within 6 hr from all tissues except liver, in which the elimination of the drug was slower than in other tissues. Radioactivity was undetectable in all organs examined 24 hr after administration.

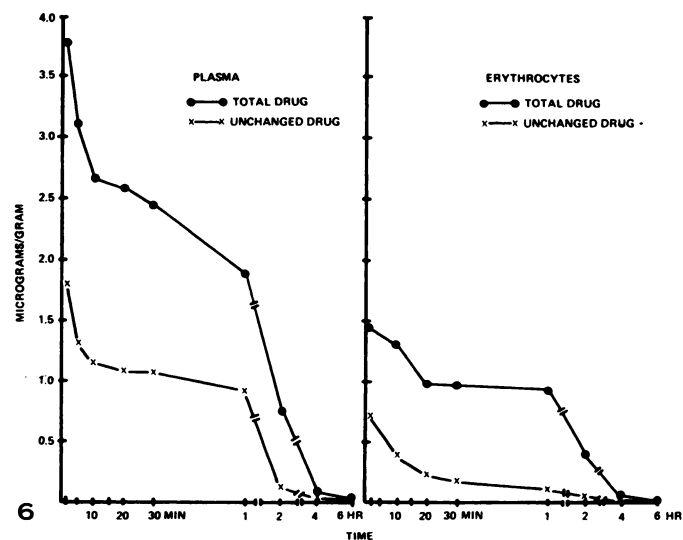
In another series of experiments, tissue distribution and metabolism of the drug was studied after i.v. administration. The drug dose (1.75 mg, 79 μCi /kg) was 170-fold lower than that used i.p. The data are summarized in Charts 4, 5, and 6. Thirty % of the drug disappeared from the plasma within 10 min and 80% disappeared within 2 hr, showing an initial rapid phase of disappearance followed by a slow phase of disappearance. The earlier phase probably represents the rapid entry of the drug into body tissues, whereas the 2nd phase represents the excretion and metabolism of the drug. Within 2 min after i.v. administration, nearly one-half of the drug present in the circulation was in the form of metabolites. In most of the tissues the drug attained peak concentrations within 15 min and was extensively metabolized (41 to 74%). The half-life values of the total radioactivity and the unchanged drug in tissues after i.p. administration were 191 to 300 and 180 to 191 min, respectively. The corresponding values after i.v. administration were 60 to 90 and 10 to 80 min, respectively. After the i.v. route, the half-life values of the total drug were severalfold higher than that of the unchanged drug in all tissues except kidney, tumor, and plasma. It is likely that the difference observed after the i.v. route and not after the i.p. route might be due not to the difference in route of



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5



6

Charts 4, 5, and 6. Levels of total and unchanged drug in tissues after i.v. administration. Tumor-bearing rats were given APH- ^{14}C , 1.75 mg (79 μCi)/kg i.v. and 2 rats were sacrificed at various time intervals.

administration but to that in total drug administered and the drug levels attained in tissues. The half-life of the unchanged drug in tumor was significantly greater than that in all of the tissues after the i.v. route of administration.

Excretion Studies. The urinary excretion data (Chart 3) show that more than one-half (55%) of the radioactivity injected into rats appeared in urine within 6 hr and nearly 92% appeared by the end of 24 hr, indicating that urinary excretion is the primary route of excretion of the drug and its metabolites. Unchanged drug in urine was found to be 2.5 to 4% of the total drug equivalents excreted in 24 hr which confirmed the results of the tissue distribution studies (Charts 1 to 6). Results from 2-dimensional paper chromatography of urine from APH-treated rats are presented in Table 1. It can be seen that there are at least 2 major metabolites appearing in the urine, one with an R_F of 0.75 and the other with an R_F of 0.42 in butanol:acetic acid: water. Although the 2 major metabolites have not been identified, Metabolite 2 (Table 1) has an R_F value corresponding to that of picolinic acid (Table 2) in both solvent systems.

DISCUSSION

The distribution of APH- ^{14}C in tissues after an optimum i.p. therapeutic dose and a lower dose given i.v. was studied in rats bearing an 8- to 9-day-old Walker carcinosarcoma 256 tumor. No preferential uptake of the unchanged drug or metabolite was found in any organ. The uptake of total radioactivity was relatively greater in kidney than other tissues, which may be due to the fact that most of the drug-derived radioactivity was excreted in urine. The amount of the unchanged drug was somewhat greater in intestine than in other organs, which may suggest lesser degradation of the drug in the intestine. The good uptake of the drug in brain is consistent with the fact that the drug is

Table 2
 R_F values of APH and related compounds

Compounds	Butanol:water (50:8)	Butanol:acetic acid:water (4:1:5)
APH (NSC 68626)	0.58	0.68
Picolinaldehydeoxime (NSC 66484)	0.68	0.81
2-Picolinoylhydrazine (NSC 81875)	0.60	0.68
Picolinamide (NSC 52473)	0.68	0.75
Picolinic acid	0.11	0.43
Metabolite 1		0.75
Metabolite 2		0.42

lipophilic as shown by its solubility in chloroform. It is likely that the reversible neurotoxicity observed in humans treated with drug (7) may be related to the high uptake and rapid disappearance of the drug in brain. Various B_6 vitamins, given intracerebrally, have been shown to protect rats from the neurotoxicity of 1, 1-dimethylhydrazine (3). Toxicity in humans from another methylhydrazine derivative (procarbazine) could also be prevented by prophylactic administration of vitamin B_6 (5). If the neurotoxicity of APH is the function of the hydrazine moiety, vitamin B_6 may prove useful in alleviating this neurotoxicity. The half-life and the concentration of the total-drug equivalents, as well as the unchanged drug in plasma, were comparable with all other tissues, which suggests that the drug is easily diffusible and not selectively accumulated by tissues to any significant extent. Although the drug is extensively metabolized (58 to 88%) within 2 to 3 hr after i.p. administration, the half-life values of the unchanged drug and the total radioactivity were not markedly different, indicating that the rate of metabolism decrease paralleled the decrease in drug concentration in tissues. In less than 2 min after i.v. administration more than one-half of the drug was found in plasma as metabolites. In some tissues up to 78% of the drug was present in the metabolized form within 30 min after i.v. administration.

Except in kidney and tumor, the half-life of the total radioactivity in all the organs after i.v. administration was 2- to 9-fold higher than the half-life of the unchanged drug. The difference was highest in the liver and erythrocytes. The half-life of the unchanged drug was 10 min, while the half-lives of the total drug were 80 and 90 min, respectively, suggesting that these organs may be the major sites of metabolism of the drug.

In clinical studies (7) the half-life of the drug in plasma based on the 3H -labeled drug (3H in acetyl moiety) was found to be 6.5 hr, somewhat longer than that observed in rats in the present study. This difference could possibly be attributed to difference in route of administration of the drug. In the clinical study the route of administration was not i.p. but either p.o. or i.v. The excretion data from the clinical studies indicated recovery of 44% of the total radioactivity within 24 hr. The lower recovery relative to that found in the present study may be partly due to the possible loss of the acetyl group containing radioactivity, which may then be retained by the body for longer periods,

Table 1
Separation of APH and its metabolites by 2-dimensional paper chromatography

Metabolites ^a	Butanol:acetic acid:water (R_F)	cpm total urine (31 ml)	% of whole urine cpm (48.3×10^6)
1	0.75	13.0	27.0
2	0.42	4.29	8.9
3	0.42	0.1	0.2
4	0.31	0.038	0.07
5	0.25	0.041	0.09
6	0.71	0.67	1.4
Unchanged drug	0.70	1.2	2.5
Total			40.16

^a A rat bearing Walker tumor was given an injection of 301.8 mg APH- ^{14}C (specific activity, 0.05 mCi/mmol)/kg, and the urine was collected for 24 hr. Twenty μ l of the urine were spotted on Whatman No. 1 chromatographic paper and developed with butanol:water (50:8). The R_F value of the unchanged drug was 0.58 and that of the metabolites was less than 0.3. The chromatogram was again developed in another direction with butanol:acetic acid:water (4:1:5). The spots visualized by uv were eluted and counted for radioactivity.

and partly due to a species difference. Our studies have shown that the drug is extensively metabolized in the rat and the major route of excretion is kidney. Although 55% and more than 90% of the injected radioactivity appeared in urine within 6 and 24 hr, respectively, the amount of unchanged drug in urine was less than 5%. Urinary excretion studies using a fluorometric method (1) in humans (7) have shown that 50% of the dose appeared in urine within 6 hr, which is close to our observation. Though the difference in routes of administration used in our studies and the clinical studies may have some influence on the rate of urinary excretion of the drug.

The metabolism of hydrazines and hydrazides has been the subject of extensive research in the past 20 years (2). Compounds structurally related to APH, *e.g.*, INH and *N*¹-acetyl-*N*²-isonicotinylhydrazine, are known to follow 2 primary routes of detoxification: hydrolysis and direct conjugation.

Although the major mode of detoxification of INH in man is direct conjugation, the extremely labile C—N bond between the aromatic carbonyl and the hydrazine break results in the formation of isonicotinic acid and the hydrazine moiety (2). In dogs, rats, and rabbits, hydrolysis of INH and *N*¹-acetyl-*N*²-isonicotinylhydrazine has been found to be the primary mode of detoxification. Isonicotinic acid may be excreted either as free acid or as conjugates of glycine, as in man and rats (4, 9, 13), or glucuronide, as in rabbits (8). The hydrazine moiety may be excreted either in monacetyl or diacetyl forms (11, 12). Comparison between the tissue distribution and excretion of INH (10) and APH indicates that both compounds are taken up by all the tissues in comparable amounts and that no tissue showed any preferential accumulation of these drugs. Also, both drugs were extensively metabolized *in vivo* and excreted primarily via the kidney. On the basis of these observations it may be inferred that APH is hydrolyzed in rats, liberating picolinic acid and hydrazine moieties that are subsequently excreted, partly free and partly as conjugates. The probable detection of free picolinic acid in urine supports this possibility. Further studies are in progress to determine the

transformation and conjugation products of APH in various species.

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