comparable to that determined by standard equilibrium dialysis or ultrafiltration techniques. The microultrafiltration technique is rapid and requires a total of only 4 ml of plasma for binding determinations at 10 drug concentrations. The technique, however, does require a sufficiently sensitive analytical method to detect the amount of free drug in 20–100 μl of plasma water.

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Effect of Aspirin on Biotransformation of ^{14}C-Acetaminophen in Rats

BARRY H. THOMASˣ, WALTER ZEITZ, and BLAKE B. COLDWELL

Abstract □ Aspirin (210 mg/kg po) administered to rats concomitantly with ^{14}C-acetaminophen (150 mg/kg po or 75 mg/kg iv) caused: *(a)* a reduction in the rate but not in the extent of acetaminophen absorption from the GI tract; *(b)* an enhanced blood level of radioactivity during the postabsorptive phase, irrespective of the route of administration of acetaminophen; *(c)* large changes in the proportions of acetaminophen metabolites and acetaminophen excreted in the urine; *(d)* a reduction in sulfate conjugation; and *(e)* an increase in glucuronide and mercapturate conjugation. These effects were not significantly altered by caffeine or codeine.

Keyphrases □ Aspirin—effect on acetaminophen absorption, rat □ Biotransformations, rat—acetaminophen, acetaminophen–aspirin interaction □ Acetaminophen—effect of aspirin on absorption and biotransformation, rat

Aspirin has been shown to alter the metabolism of phenacetin (1) and salicylamide (2) in humans, but two studies using conventional estimation procedures failed to detect any effect on the metabolism of acetaminophen (3, 4). The acetaminophen metabolites estimated by these authors were the glucuronide and sulfate conjugates.

Since acetaminophen is used in some analgesic formulations that include aspirin, it was considered desirable to investigate any possible effect on acetaminophen metabolism. The use of ^{14}C-ring-labeled acetaminophen in the rat permitted the estimation of a metabolite not included in the earlier studies in humans. The dose used was chosen to represent abusive use in humans. In an unpublished 200-day chronic toxicity study in rats, a mixture of aspirin, acetaminophen, caffeine, and codeine at the dose used in the present study produced some renal pathology but did not affect weight gain. The LD_{50} for acetaminophen in the rat is 3.7 g/kg (5) and for aspirin it is 1.48 g/kg (6). Based on data of Clark *et al.* (7), it would appear that a 25-g dose of acetaminophen in humans (approximately 0.36 g/kg) is lethal in about 50% of the cases. In view of the much lower toxicity of acetaminophen in rats compared to humans, it was considered reasonable to use a dose near the lethal dose in humans but well below the lethal dose in rats.

EXPERIMENTAL

^{14}C-Acetaminophen (*N*-acetyl-*p*-aminophenol-ring-UL-^{14}C), specific activity 17.24 μCi/mg, was custom synthesized[1]. Aspirin[2], caffeine citrate[3], codeine phosphate[4], and unlabeled acetaminophen[5] were obtained from commercial sources.

Male Wistar rats, 200–250 g, were deprived of food but not of water for 16 hr prior to dosing. In the oral dosing experiments, the rats were randomly divided into three treatment groups of six rats per group. Group 1 received a mixture of aspirin (210 mg/kg), acetaminophen (150 mg/kg), caffeine citrate (30 mg/kg), and codeine phosphate (7.5 mg/kg) in 0.25% gum tragacanth (Mixture II). Group 2 received a mixture of aspirin (210 mg/kg) and acetaminophen (150 mg/kg) in 0.25% gum tragacanth (Mixture I). Group 3 received acetaminophen (150 mg/kg) in 0.25% gum tragacanth. All three treatments were given orally as single doses in a volume of 10 ml/kg. The dose of ^{14}C-acetaminophen was 25 μCi/kg. The formu-

[1] Mallinckrodt, St. Louis, MO 63160. UL = uniformly labeled.
[2] J. T. Baker Chemical Co., Phillipsburg, NJ 08865
[3] K & K Laboratories Inc., Plainview, NY 11803
[4] B. D. H. Canada Ltd., Toronto, Canada.
[5] Matheson, Coleman & Bell, Norwood, Ohio.

Table I—Blood Concentration of Radioactivity, Expressed as Micrograms of Acetaminophen per Milliliter of Blood, in Groups of Six Rats Dosed Orally with ^{14}C-Acetaminophen (150 mg/kg)[a]

Hours	Acetaminophen	Treatments[b] Mixture I	Mixture II
0.25	98.49 ± 6.03	77.78 ± 5.65 $p < 0.05$	45.30 ± 3.07 $p < 0.001$
0.5	133.18 ± 3.71	110.73 ± 3.52 $p < 0.005$	79.76 ± 2.66 $p < 0.001$
1.0	106.99 ± 4.10	95.14 ± 3.14 $p < 0.05$	82.30 ± 4.34 $p < 0.005$
2.0	51.44 ± 2.93	54.69 ± 2.83 N.S.	56.04 ± 4.45 N.S.
4.0	9.75 ± 1.43	21.94 ± 1.35 $p < 0.001$	25.42 ± 2.59 $p < 0.001$
6.0	6.19 ± 0.62	14.81 ± 2.19 $p < 0.005$	8.80 ± 1.65 N.S.
12.0	4.82 ± 0.28	8.82 ± 0.55 $p < 0.001$	5.68 ± 0.78 N.S.
24.0	2.11 ± 0.09	2.81 ± 0.29 $p < 0.05$	2.39 ± 0.39 N.S.

[a] Values are means ± *SE*, and the *p* values represent the probability when compared with the control group (acetaminophen). [b] Mixture I = aspirin and acetaminophen; and Mixture II = aspirin, acetaminophen, caffeine, and codeine.

lation of Mixture II corresponds to popular phenacetin-containing analgesic tablets sold over-the-counter in Canada, with acetaminophen being substituted for phenacetin.

Duplicate blood samples (10 μl) were collected from the tail at 0.25, 0.50, 1, 2, 4, 6, 12, and 24 hr after dosing. The blood was digested and counted in a liquid scintillation counter as previously described (8). Urine was also collected at 12 and 24 hr after dosing and estimated for radioactivity (8). Some urines were incubated overnight at 37° with an equal volume of β-glucuronidase[6] or with β-glucuronidase–sulfatase[7]. When the mixture was used, 0.5 ml of 0.2 *M* acetate buffer (pH 5.0) was added to 0.5 ml of urine followed by 50 μl of β-glucuronidase–sulfatase.

When acetaminophen was administered intravenously, two treatment groups were used. Group 1 received an oral dose of aspirin (210 mg/kg) in 0.25% gum tragacanth, and Group 2 received a similar dose of the vehicle alone. After 30 min, both groups were

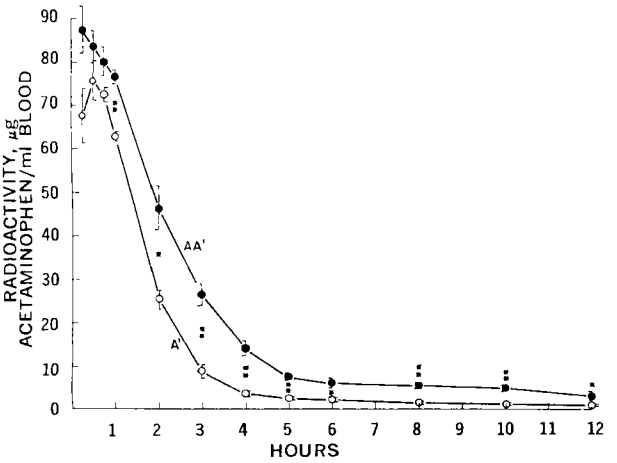

Figure 1—*Blood concentration of radioactivity expressed as micrograms of acetaminophen per milliliter of blood. Mixture I is aspirin and acetaminophen. Values are means* ± SE *from four rats per group dosed intravenously with* ^{14}*C-acetaminophen (75 mg/kg). Key:* ○, *acetaminophen alone;* ●, *Mixture I;* *, p < *0.05; and* *, p < *0.005.*

[6] Ketodase, Warner-Chilcott, Morris Plains, N.J.
[7] Glusulase, Endo Laboratories, Inc., Garden City, NY 11530

Table II—Effect of β-Glucuronidase–Sulfatase on the Paper Chromatography of 0–12-hr Urine from Single Rats Dosed Orally with Either Acetaminophen or Mixture I

Drug Treatment[a]	Enzyme Treatment	Chromatography Peaks[b], % I	II	III	IV
Acetaminophen	—	22.5	7.9	64.1	5.5
	β-Glucuronidase	1.7	8.4	64.0	26.0
	β-Glucuronidase–sulfatase	2.9	7.9	0	89.3
Mixture I	—	32.2	16.1	38.3	13.4
	β-Glucuronidase	3.2	16.4	37.4	42.9
	β-Glucuronidase–sulfatase	4.0	16.4	0	79.6

[a] Abbreviations as in Table I. [b] I = glucuronide, II = mercapturate?, III = sulfate, and IV = unchanged drug.

injected in the saphenous vein with ^{14}C-acetaminophen (75 mg/kg, 25 μCi/kg) in a volume of 1.0 ml/kg of 50% ethanol under light ether anesthesia. Duplicate blood samples (10 μl) were collected from the tail at 0.25, 0.50, 0.75, 1, 2, 3, 4, 5, 6, 8, 10, and 12 hr after the dose of acetaminophen and estimated for radioactivity. Urine was also collected up to 12 hr.

Metabolites of acetaminophen in urine were determined using the alkaline paper chromatography system described by Shahidi (9, System C). Urine (10–25 μl) was applied as a band to strips of chromatography paper[8]. Radioactivity on the developed chromatograms was detected by serial sectioning into 1-cm strips and counting in a liquid scintillation counter. A few of the urines were also chromatographed on a resin column[9] using a scaled down version of that described by Jagenburg *et al.* (10). The column dimensions were: diameter, 0.9 cm; height, 45 cm; and total bed volume, 30 ml.

RESULTS

The blood concentrations of radioactivity in the three treatment groups given ^{14}C-acetaminophen by the oral route are shown in Table I. The two groups given drug combinations had lower blood levels during the 1st hr than the group given acetaminophen alone. This effect was greater with Mixture II than with Mixture I. At 4 hr the effect was reversed with higher blood levels in the drug combination groups. The group that received Mixture I comtinued to have significantly higher blood levels than the acetaminophen

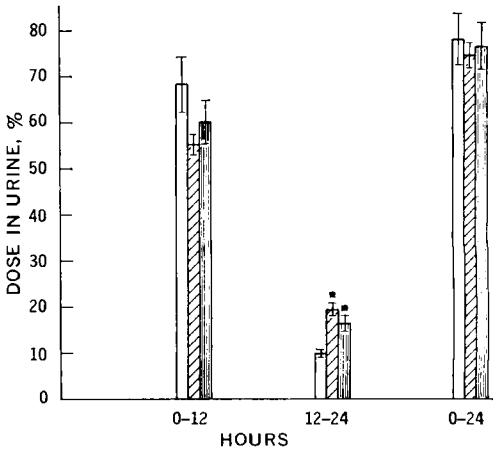

Figure 2—*Urinary excretion of radioactivity in rats dosed orally with* ^{14}*C-acetaminophen (150 mg/kg). Mixture II is aspirin, acetaminophen, caffeine, and codeine; and Mixture I is aspirin and acetaminophen. Values are means* ± SE *from six rats per group. Key:* □, *acetaminophen alone;* ▨, *Mixture I;* ▥, *Mixture II; and* *, p < *0.05 compared to acetaminophen alone.*

[8] Whatman No. 1.
[9] Sephadex gel G10.

Table III—Urinary Excretion of Acetaminophen and Its Metabolites in Rats Dosed Orally with ^{14}C-Acetaminophen (150 mg/kg)[a]

Hours	Metabolite	Acetaminophen	Treatments[b] Mixture I	$p<$	Mixture II	$p<$
0–12	Glucuronide	21.13 ± 0.97	34.60 ± 1.91	0.001	29.35 ± 1.61	0.005
	Mercapturate	7.46 ± 0.23	16.60 ± 0.64	0.001	17.22 ± 0.91	0.001
	Sulfate	64.27 ± 1.37	34.56 ± 1.94	0.001	40.02 ± 1.97	0.001
	Unchanged, acetaminophen	7.14 ± 0.77	14.24 ± 1.25	0.001	13.41 ± 1.75	0.01
12–24	Glucuronide	12.29 ± 0.72	16.92 ± 1.66	0.05	16.81 ± 1.24	0.02
	Mercapturate	5.07 ± 0.42	14.36 ± 1.25	0.001	9.77 ± 0.75	0.001
	Sulfate	80.04 ± 0.94	64.45 ± 1.84	0.001	67.03 ± 2.24	0.001
	Unchanged, acetaminophen	2.60 ± 0.59	4.27 ± 1.14	N.S.	6.38 ± 3.08	N.S.

[a] The proportion of each metabolite is expressed as a percentage. Values are means ± *SE*, and the *p* value represents the probability when compared with the control group (acetaminophen). [b] Abbreviations as in Table I.

alone group throughout the 4–12-hr period studied. To avoid complications due to interaction between the drugs during absorption from the GI tract, acetaminophen was given intravenously. The blood profiles shown in Fig. 1 indicate that aspirin does increase the blood level of radioactivity. When these data were plotted on semilogarithmic graph paper, it was found that elimination fell into two phases in both groups. There was rapid elimination up to 4 hr followed by a slower rate from 5 to 12 hr. The effect of aspirin was most apparent during the first phase (half-life: acetaminophen alone, 0.72 hr; Mixture I, 1.13 hr).

The total urinary excretion of radioactivity in the three treatment groups given oral acetaminophen is shown in Fig. 2. During the 0–12-hr period, there was no significant differences; but during the 12–24-hr period, there was a significant increase in excretion in the Mixture I and Mixture II groups compared to the controls. This effect could be due to a slower rate of absorption and/or a reduced elimination rate in the initial time period. The cumulative 24-hr excretion of radioactivity was high in all groups, indicating that absorption was essentially complete.

Paper chromatoraphy of the urines separated the radioactivity into four distinct peaks. Treatment of some urines with β-glucuronidase–sulfatase gave results such as those shown in Table II. β-Glucuronidase reduced only peak I and proportionately increased peak IV, which has the R_f of authentic acetaminophen. This indicates that peak I is largely acetaminophen glucuronide. When sulfatase was present, peak III was also eliminated; therefore, this peak is acetaminophen sulfate. Peak II was unaffected by either enzyme and its identity remains uncertain. In terms of R_f, it corresponds with that reported (10) for acetaminophen mercapturate. It also gives the color tests reported (10). The percentage of the dose excreted as unchanged drug and the three metabolites is shown in Fig. 3. The data in Table III show the proportions of each metabolite excreted in urine. There were some major shifts in metabolism due to the presence of the other drugs, but aspirin is apparently the major cause since there is little difference between the Mixture I and Mixture II groups.

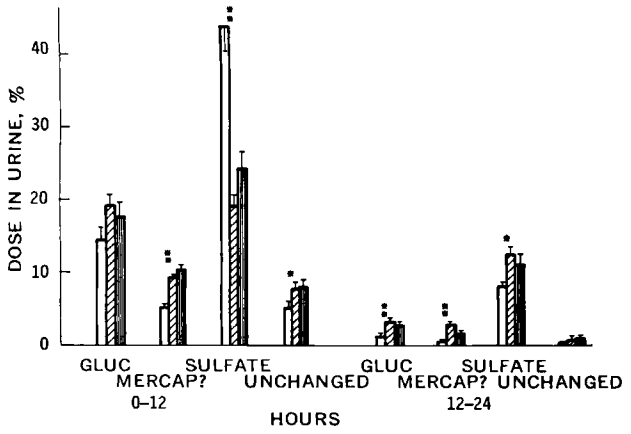

Figure 3—*Urinary excretion of acetaminophen and its metabolites in rats dosed orally with ^{14}C-acetaminophen (150 mg/kg). Mixture II is aspirin, acetaminophen, caffeine, and codeine; and Mixture I is aspirin and acetaminophen. Gluc represents the glucuronide conjugate and mercap is the mercapturate conjugate. Values are means ± SE from six rats per group. Key: □, acetaminophen alone; ▨, Mixture I; ■, Mixture II; *, p < 0.05 (compared to acetaminophen alone); and **, p < 0.005 (compared to acetaminophen alone).*

The paper chromatography data obtained from the rats dosed intravenously with acetaminophen are shown in Fig. 4. The results were similar to those obtained after oral administration except that acetaminophen glucuronide was significantly higher in the aspirin-treated rats. This experiment confirmed that the metabolic changes observed in urine were due to an effect of aspirin on metabolism and not to a slower rate of absorption.

Column chromatography confirmed the values for free acetaminophen obtained by paper chromatoraphy. A small amount of a metabolite described by Jagenburg and Toczko (11) as a cysteine conjugate was detected, but it constituted less than 0.2% of the dose.

DISCUSSION

The combination of acetaminophen with aspirin, caffeine, and codeine appeared to reduce the rate of acetaminophen absorption. Aspirin alone had a similar but less marked effect. The evidence for this was the slower rise in the blood concentration of radioactivity in the Mixture I and Mixture II groups compared to acetaminophen alone. Despite the slower rate, the total absorption was not diminished since there was a high recovery of radioactivity in the 24-hr urines from all three treatments.

During the postabsorptive phase, the blood level of radioactivity tended to be higher in the groups that received the drug combinations, particularly the Mixture I group. When acetaminophen was given intravenously, the same effect on the blood level was seen. It is deduced from these data that aspirin reduced the elimination rate of acetaminophen during the first few hours after dosing.

Over the urine collection periods studied, the other drugs had

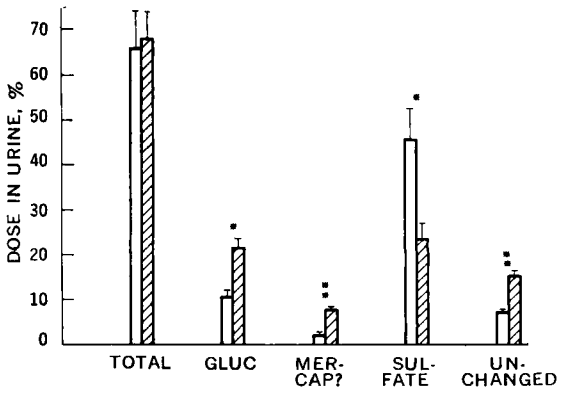

Figure 4—*Paper chromatography data obtained from rats dosed intravenously with acetaminophen. Key: □, acetaminophen alone; ▨, Mixture I; *, p < 0.05 (compared to acetaminophen alone); and **, p < 0.005 (compared to acetaminophen alone).*

only a slight effect on the total renal excretion of radioactivity. When given orally, there was an increase in the 12–24-hr period excretion in the Mixture I and Mixture II groups, but this could have been due to delayed absorption. It is possible that there was reduced excretion during the first few hours which was not apparent by 12 hr when the urine was collected.

The most significant effect of aspirin was on the proportions of the major metabolites of acetaminophen excreted in urine. This effect was seen whether acetaminophen was given orally or intravenously. The principal change was the reduction in both the amount and the proportion of acetaminophen sulfate. The increased proportions of free acetaminophen and the glucuronide and mercapturate metabolites could well be consequences of diminished sulfate conjugation. The reason for this effect of aspirin is not immediately apparent, since salicylate is not known to be conjugated with sulfate; therefore, competition for the available sulfate was not possible. However, Boström *et al.* (12) showed that salicylate inhibits the biosynthesis of mucopolysaccharide sulfates and the excretion of ester sulfates in urine. These investigators suggested that the known oxidative uncoupling action of salicylate reduced the supply of adenosine triphosphate and, hence, active sulfate (3′-phospho-5′-adenosinephosphosulfate), which is necessary for sulfate conjugation. It is possible, therefore, that the reduced amount of acetaminophen sulfate could be due to less active sulfate being available in the aspirin-treated rats.

Levy and Regardh (3) and Amsel and Davison (4) did not see any changes in acetaminophen sulfate in human urine when doses of aspirin in the high therapeutic range were given. This could be due to sulfate conjugation being quantitatively less important in humans than in rats or to the doses being less than abusive. The increased excretion by the aspirin-treated rats of a metabolite assumed to be acetaminophen mercapturate could be toxicologically important since this type of metabolite usually arises from a highly reactive precursor. In humans, both the mercapturate (10) and its immediate precursor, the cysteine conjugate (11), are found in urine after administration of normal doses of acetaminophen. In the rat, very little of the cysteine conjugate is found. If the greater excretion of unchanged acetaminophen in urine represents higher levels in the body, the duration of action of acetaminophen will be longer when aspirin is also administered.

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Field Desorption Mass Spectrometry of Azathioprine and Its Metabolites

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Abstract □ The field desorption mass spectra of azathioprine, I, and some of its metabolites, II–IV, were investigated and compared to their electron impact spectra. The field desorption spectra were shown to be especially well suited for the determination of molecular weights in drug metabolism studies of drugs conjugated with amino acids and peptides. This technique was applied to a metabolite of azathioprine isolated from rat urine and proved to be useful.

Keyphrases □ Azathioprine and metabolites—field desorption mass spectra, compared to electron impact spectra, application to molecular weight determinations □ Mass spectroscopy—comparison of field desorption and electron impact spectra, azathioprine and metabolites, application to molecular weight determinations □ Field desorption mass spectra—azathioprine and metabolites, compared to electron impact spectra, application to molecular weight determinations

Azathioprine[1] (I), [(1-methyl-4-nitroimidazol-5-yl)thio]purine, is the most widely used immunosuppressive agent in clinical organ transplantation. The metabolism of I has been the subject of extensive studies (1–7). When the metabolic fate of the methylnitroimidazole moiety of ^{14}C-azathioprine, labeled in the imidazole ring, was investigated (3, 5, 7), evidence was found that nucleophilic attack occurred on the nitromethylimidazole ring *in vivo*, leading to the formation of 5-substituted derivatives. In recent studies in the rat (3, 6), the following metabolites of azathioprine were characterized: 1-methyl-4-nitro-5-(*S*-glutathionyl)imidazole (II), 1-methyl-4-nitro-5-(*N*-acetyl-*S*-cysteinyl)imidazole (III), and 1-methyl-4-nitro-5-carboxymethylaminoimidazole (IV).

The predominant analytical methods used to iden-

[1] Imuran, Burroughs Wellcome Co.