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Drug Interactions. I. Effects of Repeated Administration of Combined Analgesic on Its Pharmacological Activity and on Hepatic Drug-metabolizing Enzyme Activities in Mice[1]

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In order to elucidate the interactions of the components of an aminopyrine (28.9 mg)–phenacetin (57.8 mg)–phenobarbital (13.3 mg) preparation on repeated administration (100 mg/kg of body weight) to mice, the analgesic activity of the preparation, the hepatic drug-metabolizing enzyme activities and the plasma concentrations of phenobarbital, phenacetin and acetaminophen in mice dosed repeatedly for 7 days were estimated in comparison with a single dose group. The analgesic activity was significantly decreased and the liver drug-metabolizing enzyme activities were markedly enhanced after the repeated treatment as compared with the results obtained following a single dose. The plasma levels of phenacetin were markedly lower at both 45 and 90 min in the repeated dose group, while the levels of acetaminophen and its glucuronide were higher in mice dosed repeatedly than in the single dose group 45 min after intraperitoneal injection, but lower after 90 min. The plasma concentrations of phenobarbital 45 and 90 min after the combined drug were not significantly different in the single and repeated dose groups. When the induction rate of the drug-metabolizing enzymes by the combined drug was compared with that by phenobarbital alone, there was a clear-cut correlation ($r=0.986$ for repeated dosing and $r=0.985$ for a single dose). This strongly suggests that phenobarbital in the combined analgesic induced metabolic inactivation of the other component drugs without significant enhancement of its own metabolism, while aminopyrine and phenacetin had no such effect.

Keywords——drug interactions; drug-metabolizing enzyme activities; analgesic activity after repeated administration; induction of drug-metabolizing enzymes; drug-metabolizing enzyme activities after repeated administration

Analgesics are often used in combination with other analgesics or sedatives in drug preparations designed to give more effective pain relief than is obtainable with one of the agents alone. Barbiturates are known to enhance the analgesic actions of salicylate, pyrazolone and *p*-aminophenol derivatives, and short-acting barbiturates are often given in combination with analgesics for the relief of pain.[3] Thus, an aminopyrine–phenacetin–barbiturate preparation is widely used for patients with migraine and neuralgia. However, analgesic abuse may result in adverse reactions and noxious effects. In addition, interactive effects of the component drugs have been found at therapeutic doses of the preparations, which can result in ineffective therapy, exaggerated therapeutic responses or even toxic responses. During the past twenty years many hundreds of drugs and other xenobiotics have been shown to induce hepatic microsomal drug-metabolizing enzymes,[4] with consequent effects on the rates

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3) H. Itoh, "Yakurigaku," 4th ed., Eikodo, Tokyo, 1972, pp. 105—111.
4) A.H. Conney and J.J. Burns, *Adv. Pharmacol.*, **1**, 31 (1962); M.G. MacDonald, D.S. Robinson, D. Sylwester, and J.J. Jaffe, *Clin. Pharmacol. Ther.*, **10**, 80 (1969); G.J. Mannering, "Selected Pharmacological Testing Methods," ed. by A. Burger, Marcel Dekker, New York, 1968, pp. 51—119.

of drug metabolism and the pharmacological activities of therapeutic agents.[5] Numerous reports on such drug interactions have been cited in reviw articles.[6]

The present study was designed to elucidate the interactions of aminopyrine–phenacetin–phenobarbital preparation on repeated administration to mice. The analgesic activity, hepatic drug-metabolizing enzyme activities and plasma concentrations of the drugs and their metabolites were determined after single and repeated administrations, and the effect of repeated administration of the combined analgesic on the pharmacological action and hepatic metabolism is discussed.

Experimental

Materials——Nicotinamide adenine dinucleotide phosphate (NADP), reduced nicotinamide adenine dinucleotide phosphate (NADPH) and cytochrome c were purchased from Sigma Chemical Co. Glucose 6-phosphate (G-6-P) dehydrogenase [EC 1.1.1.49] and G-6-P were obtained from Oriental Yeast Co., Ltd. β-Glucuronidase [EC 3.2.1.31] was obtained from P-L Biochemicals Inc. Aniline and aminopyrine used as substrates for the enzyme reaction were subjected to redistillation or recrystallization, respectively.

Animals and Treatment——Male ddy mice weighing 19—20 g were used throughout. The mice were divided at random into 3 groups, each consisting of 6—8 mice. Food was provided *ad libitum*. A) Controls were treated for 7 days with daily intraperitoneal (*i.p.*) injections of saline (Control). B) Animals were treated with a single injection of a combined analgesic (13.3 mg of phenobarbital, 28.9 mg of aminopyrine and 57.8 mg of phenacetin/kg of body weight) or the individual components alone (Single). C) Animals were treated for 7 days with daily *i.p.* injections of the analgesic (100 mg/kg of body weight/day) or the individual components (in the same doses as in B) alone. All the drugs were dissolved in 20% propylene glycol-saline mixture.

Measurement of Analgesia——Analgesia was estimated by both the acetic acid-writhing method[7] and the hot plate method.[8] The time it took for the mice to lick either hind leg or to jump on a hot plate at $57 \pm 0.5°$ was used as the response time. Analgesic activity was measured 30 min after administration of the drug (100 mg/kg of body weight) and at 15 min intervals for 75 min. For the acetic acid-writhing method, mice were injected (*i.p.*) with 0.8% acetic acid in water and from 30 min after the drug injection the frequency of writhing syndrome was counted at 10 min intervals.

Determination of Phenobarbital, Phenacetin and Acetaminophen in Plasma——Blood specimens were collected from the mice 45 or 90 min after the injection of the combined analgesic (the final dose was 200 mg/kg of body weight for an exact determination of the drugs). The plasma samples were obtained from combined blood in each group. Phenobarbital in plasma was estimated according to the method of Nishina *et al.*[9] Phenacetin in plasma was estimated by the method of Vesell *et al.*,[10] by gas–liquid chromatography (GLC) on a Shimadzu GC-4BM gas chromatograph with a hydrogen flame ionization detector using caffeine as an internal standard and a 3 mm × 2 m glass column packed with 5% Thermon 1000 on Chromosorb WAW. Acetaminophen in plasma, with salicylamide added as an internal standard, was extracted twice with ether according to the method of Evans and Harbison.[11] After evaporating off the solvent, acetaminophen dissolved in 50 μl of ethyl acetate was trifluoroacetylated with trifluoroacetic anhydride. To determine the total amount of acetaminophen present, 2.1 mg (3000 unit) of β-glucuronidase was incubated with the plasma at pH 5 for 18 hr at 37°. This amount of the enzyme ensured complete hydrolysis of the conjugate.[12] After hydrolysis, the sample was processed as described above and separated on a 3 mm × 2 m glass column packed with 15% Silicon GE SE-30 on 60—80 mesh Shimalite W.

Preparation of Liver Soluble Fractions——Male mice were starved for 24 hr and sacrificed by decapitation 18—20 hr after the final administration of saline or the drug. The liver was thoroughly perfused *in situ* with 0.9% NaCl solution. The liver was excised and chopped up with a razor, then homogenized with 4

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6) A.G. MacGregor, *Proc. R. Soc. Med.*, **58**, 143 (1965); E.A. Hartshorn, *Drug Intell.*, **2**, 174 (1968); D.L. Azarnoff and A. Hurwitz, *Pharmacol. Physic.*, **4**, 1 (1970); D.W. Yesair, F.J. Bullock, and J.J. Coffey, *Drug Metab. Rev.*, **1**, 35 (1972); V.M. Rosenoer and G.M. Gill, *Med. Clin. N. Am.*, **56**, 585 (1972); "Evaluations of Drug Interactions," 2nd ed., by American Pharmaceutical Association, Washington, D.C., 1976.
7) K. Tsuda and H. Nogami (ed.), "Yakko No Hyoka (1), Yakuri Shikenho," Chijin Shokan, Tokyo, 1971, pp. 226—227.
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10) E.S. Vesall, G.T. Passananti, P.A. Glenwright, and B.H. Dvorchik, *Clin. Pharmacol. Ther.*, **18**, 259 (1975).
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volumes of 1.15% KCl solution in a glass Potter homogenizer equipped with a Teflon pestle. The homogenate was centrifuged at 9000×*g* for 25 min. The supernatant fractions obtained were used in this experiment.

Assays of Drug-metabolizing Enzyme Activities——The demethylation of aminopyrine was assayed at 37° by the method of Ariyoshi and Takabatake[13] and formaldehyde formed was determined by the method of Nash.[14] Aniline *p*-hydroxylase activity of the fractions was measured according to the method of Ikeda.[15] NADPH cytochrome c reductase activity was assayed by the method described by Slater and Sawyer.[16] Cytochrome P-450 (P-450) in the 9000×*g* soluble fractions was routinely determined by the method of Omura and Sato.[17]

Protein Determination——Protein concentration was estimated by the procedure described by Lowry *et al.*[18]

Statistical Analysis——The data were compared by an analysis of variance. When the analysis indicated that a significant difference existed, the means of the treated groups were compared with the control mean by Student's *t*-test with $p \leq 0.05$ as the criterion of significance. Correlation analyses were carried out by the method of least-squares linear regression. Correlation coefficients were examined for significance ($p \leq 0.05$) by the *t*-test.

Results

Analgesic Activity Following Single or Repeated Administration of the Combined Analgesic

Each mouse received a single or 7 days' treatment with the combined analgesic and, following the final administration, the analgesic response was measured. The results are shown in Figs. 1 (the acetic acid-writhing method) and 2 (the hot plate method). The analgesic activity measured by the acetic acid-writhing method was significantly decreased after repeated treatment as compared with the result obtained following a single administra-

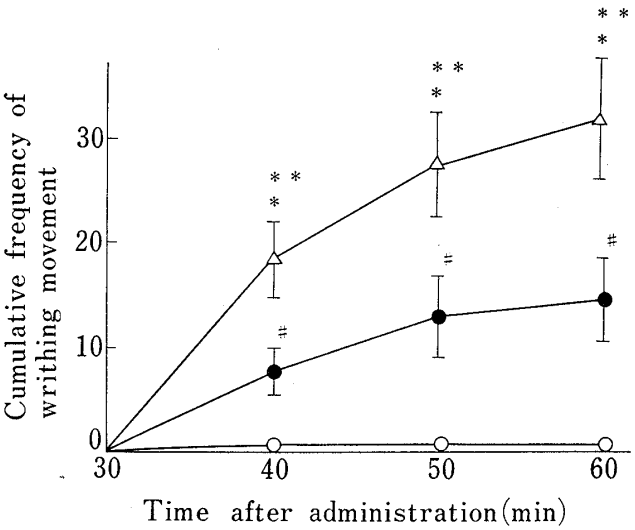

Fig. 1. Analgesic Activity determined by the Writhing Method after Single and Repeated Administrations of the Analgesic

The writhing reaction was measured after administration of the analgesic (100 mg/kg body weight, *i.p.*). Each point represents the mean ±S.E.
Repeated, ●—●; single, ○—○; control, △—△.
* $p<0.05$ in repeated *vs.* control.
♯ $p<0.01$ in repeated *vs.* single.
** $p<0.001$ in single *vs.* control.

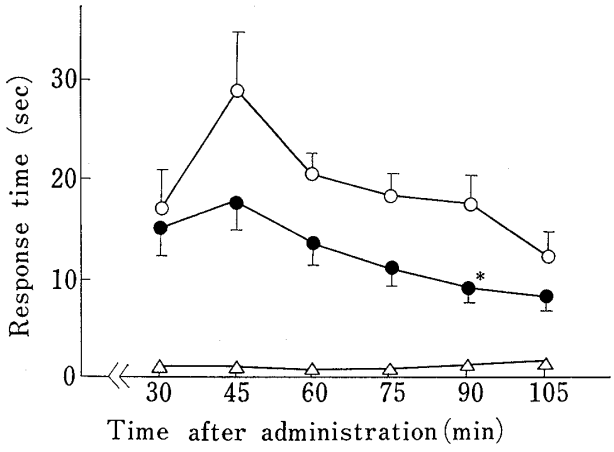

Fig. 2. Analgesic Activity determined by the Hot Plate Method after Single and Repeated Administrations of the Analgesic

The reponse was measured after administration of the analgesic (100 mg/kg body weight, *i.p.*). Each point represents the mean ±S.E.
Repeated, ●—●; single, ○—○; control, △—△.
$p<0.001$ in control *vs.* repeated or single in all groups.
* $p<0.02$ in repeated *vs.* single.

13) T. Ariyoshi and E. Takabatake, *Life Sci.*, **9**, 361 (1970).
14) T. Nash, *Biochem. J.*, **55**, 416 (1953).
15) M. Ikeda, *J. Biochem.* (Tokyo), **55**, 231 (1964).
16) T.F. Slater and B.C. Sawyer, *Biochem. J.*, **111**, 317 (1969).
17) T. Omura and R. Sato, *J. Biol. Chem.*, **239**, 2370 (1964).
18) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

tion. The mice that received a single administration of the analgesic showed little response owing to its strong analgesic action. In general, the response time by the hot plate method was more shortened after repeated administration. When the hot plate method was used, the maximal analgesic effect was seen 45 min after administration. This indicates relatively rapid absorption of the analgesic. Using the area under the response time curve (30 to 105 min) as an index of the bioavailability, repeated administration of the combined analgesic diminished the bioavailability to about 64% compared to a single administration. These results indicate that repeated treatment of this combined analgesic for 7 days leads to a drastic decrease in the analgesic effect and induces the development of apparent tolerance to the drug.

When the analgesic activity of phenobarbital (13 mg/kg of body weight) alone was estimated in comparison with that of the combined drug, no analgesic activity of the agent was found by either method.

Effect of Single or Repeated Administration of the Combined Analgesic on Liver Drug-metabolizing Enzyme Activities and Liver Weight

To clarify the mechanism of the decreased analgesic activity after 7 days' treatment with the combined analgesic, the liver drug-metabolizing enzyme activities and liver weights of mice that had received the drug were estimated. As shown in Table I, a single dose of the analgesic enhanced the activities by 14—68% as compared with the control, and more significant enhancement could be seen after repeated dosing (41—116%). The enhancement of the activities after repeated administration was dramatic for aminopyrine N-demethylase and P-450. Thus, induction of liver drug-metabolizing enzymes by this analgesic is probably associated with the decrease in analgesic activity noted after repeated treatment with the combined drug. The observed difference in the controls between metabolizing enzyme activities may be due to the different periods of the assays and minor differences in the mice used.

Relative liver weights (g liver/100 g of body weight) were significantly ($p<0.01$) increased in both the single and repeated dose groups as compared with the control, but no difference between the groups was found (control; 4.85±0.09, single; 5.63±0.11, repeated; 5.66±0.19).

Table I. Effect of Single and Repeated Administrations of the Analgesic on Drug-metabolizing Enzyme Activities

Enzymes	Treatment	Activities	%
Aniline *p*-hydroxylase*	Control	0.99 ±0.05	100
	Single	1.14 ±0.04	115
	Repeated	1.59 ±0.10[a,b]	161
Aminopyrine N-demethylase*	Control	1.43 ±0.12	100
	Single	2.40 ±0.09[a]	168
	Repeated	3.09 ±0.17[a,c]	216
NADPH-cytochrome c reductase*	Control	9.3 ±0.6	100
	Single	10.6 ±0.7	114
	Repeated	13.2 ±1.0[d,e]	141
Cytochrome P-450**	Control	0.094±0.006	100
	Single	0.119±0.004[f]	127
	Repeated	0.173±0.011[a,g]	184

Each value represents the mean of 5—8 mice±S.E.
* The activities of enzymes are expressed as nmol of product per min per mg of protein.
** The content of cytochrome P-450 is expressed as nmol per mg of protein.
a) $p<0.001$ in control *vs.* single or repeated.
b) $p<0.01$ in single *vs.* repeated.
c) $p<0.02$ in single *vs.* repeated.
d) $p<0.05$ in single *vs.* repeated.
e) $p<0.02$ in control *vs.* single or repeated.
f) $p<0.01$ in control *vs.* single or repeated.
g) $p<0.001$ in single *vs.* repeated.

Plasma Concentrations of Phenobarbital, Phenacetin, Acetaminophen and Its Glucuronide after Single or Repeated Administration of the Combined Analgesic

The plasma concentrations of phenobarbital in the repeated dose group at both 45 and 90 min after administration of the combined drug were only slightly lower than those in the single dose group. The levels 45 min after dosing in both groups were similar to those 90 min after dosing, suggesting extremely slow elimination of the drug. As an index of the analgesic action, the plasma levels of phenacetin and acetaminophen were measured. The plasma levels of phenacetin in mice administered repeatedly were significantly lower than in the single dose group 45 min after injection of the combined drug and fell almost to zero after 90 min. This suggested that O-dealkylation of phenacetin was enhanced in mice dosed repeatedly, as shown in Table II. On the other hand, it is noteworthy that the mice had slightly higher plasma levels of acetaminophen, an active metabolite of phenacetin, in the repeated dose group than in the single dose group 45 min after administration of the combined drug. The plasma concentrations of acetaminophen glucuronide in the repeated dose group, however, were markedly higher than those in the single dose group 45 min after administration, indicating that the formation of the glucuronide conjugate was markedly enhanced by 7 days' treatment with the combined analgesic. The plasma levels of the glucuronide in the repeated dose group, however, decreased 90 min after administration as compared with those in the single dose group. These results indicate that a significant increase in dealkylation, conjugation and metabolic clearance of the component drugs occurs, probably due to enhanced activities of the drug-metabolizing enzymes, and this seems to be related to the decreased analgesic activity.

Table II. Plasma Concentrations of Phenobarbital, Phenacetin, Acetaminophen and Its Glucuronide after Single or Repeated Administrations

Treatment	Concentrations (μg/ml of plasma)							
	Phenobarbital		Phenacetin		Acetaninophen			
					Free		Glucuronide conjugate[a]	
	45 min	90 min	45 min	90 min	45 min	90 min	45 min	90 min
Single	23.89	26.00	19.53	2.43	17.05	12.40	35.75	33.08
Repeated	22.00	22.40	8.16	0.55	21.78	5.31	52.22	20.29

a) The amount of glucuronide conjugate was calculated by subtracting the amount of free acetaminophen from the total amount determined after hydrolysis of conjugates with β-glucuronidase.

Effect of Each Component of the Combined Analgesic on Drug-metabolizing Enzyme Activities

To clarify the involvement of each component in induction of the drug-metabolizing enzyme activities, the individual components were given separately to mice by single or repeated administration for 7 days at doses corresponding to the amount contained in the combined drug. As shown in Table III, phenobarbital markedly enhanced the enzyme activities, especially after repeated doses (64—142%) (for NADPH cytochrome c reductase, single; 117%, repeated; 164% as compared with the control), and aminopyrine enhanced the activities a little after a single administration (22—45%) but rather decreased them on repeated administration. Phenacetin showed little or no induction of the enzymes at the dosage tested, and the activities even appeared to decrease slightly. When the induction rate of the enzyme activities with phenobarbital was compared with that by the combined analgesic, there was a good agreement. The correlation coefficient was 0.986 ($p<0.001$) for repeated treatment with the combined drug and phenobarbital and 0.985 ($p<0.001$) for a

single treatment. This strongly suggests that the inductive effect of phenobarbital is the major factor in the decreased analgesic activity following repeated administration of the combined analgesic.

Table III. Effect of Single and Repeated Administrations of Each Component of the Analgesic on Drug-metabolizing Enzyme Activities and Relative Liver Weights

Enzymes	Treatment	Phenobarbital		Aminopyrine		Phenacetin	
		Activities	%	Activities	%	Activities	%
Aniline *p*-hydroxylase	Control	1.09 ±0.10	100	0.75 ±0.02	100	1.05 ±0.10	100
	Single	1.39 ±0.03[a]	128	0.97 ±0.05[d]	129	0.97 ±0.09	92
	Repeated	1.83 ±0.11[b,c]	168	0.84 ±0.03[a]	112	0.97 ±0.03	92
Aminopyrine N-demethylase	Control	1.52 ±0.07	100	1.52 ±0.01	100	1.52 ±0.07	100
	Single	2.72 ±0.14[d]	179	1.86 ±0.06[d]	122	1.27 ±0.13	84
	Repeated	3.67 ±0.20[b,c]	241	1.65 ±0.03[d]	109	1.42 ±0.04	93
Cytochrome P-450	Control	0.096±0.003	100	0.094±0.009	100	0.124±0.006	100
	Single	0.140±0.004[b]	146	0.136±0.009[a]	145	0.107±0.010	86
	Repeated	0.196±0.001[b,c]	204	0.116±0.004	123	0.122±0.005	98
Relative liver weights (g/100 g body weight)	Control	5.24 ±0.05	100	4.96 ±0.05	100	4.77 ±0.07	100
	Single	5.75 ±0.09[b]	110	4.81 ±0.08	99	4.49 ±0.07[a]	94
	Repeated	5.81 ±0.05[b]	111	4.95 ±0.07	100	4.84 ±0.10[e]	101

Details, see the legend to Table I. Each value represents the mean of 5—8 mice ±S.E.
a) $p<0.05$ in control *vs.* single or repeated.
b) $p<0.001$ in control *vs.* single or repeated.
c) $p<0.01$ in single *vs.* repeated.
d) $p<0.01$ in control *vs.* single or repeated.
e) $p<0.05$ in single *vs.* repeated

Discussion

Many kinds of analgesics, in single or combined forms, have been used in clinical therapy. Combinations of barbital, aminopyrine and phenacetin were found to be more effective than each drug alone.[3] However, little is known about the effect of repeated administration of the combined analgesics on the pharmacological action and on drug-metabolizing enzyme activities, or about the mechanism of the development of tolerance on repeated dosing. Assessment of the contribution of each component of the drug to the overall metabolism and pharmacological effect in combined drugs is difficult, although some studies have been with combinations of two kinds of drugs.[19]

The present study demonstrated that 7 days' treatment with this combined analgesic significantly decreased the analgesic activity (Figs. 1 and 2), mainly due to the significant inductive effect of phenobarbital, a component of the combined analgesic, on drug-metabolizing enzymes (Tables I and III). Repeated treatment with this analgesic markedly enhanced O-dealkylation of phenacetin and glucuronide conjugation of acetaminophen (Table II), consequently increasing elimination of the components and their metabolites, so that the apparent tolerance to the analgesic was marked after 7 days' treatment, whereas the effect of the inductive action after a single dose appeared to be negligible since there was only a slight induction within 2 hr after administration of the analgesic (unpublished data). In a single dose, a higher analgesic activity of the combined drug was observed 45 min after injection despite the lower blood level of acetaminophen, but there was a higher level of phenacetin

19) *a*) I.E. Leppik and A.L. Sherwin, *J. Pharmacol. Exp. Ther.*, **200**, 570 (1977); *b*) S. Ship and P. Kourounakis, *Biochem. Pharmacol.*, **26**, 467 (1977); *c*) E.S. Vesell, G.T. Passananti, and G.W. Hepner, *Clin. Pharmacol. Ther.*, **20**, 661 (1977); *d*) U. Breyer, W. Junginger, and D. Villumsen, *Biochem. Pharmacol.*, **25**, 2623 (1976).

compared with the repeated dose group. This suggests that the analgesic activity may be partially due to the pharmacological effect of phenacetin. Although the analgesic and antipyretic effects of phenacetin have been postulated to be exerted mainly through its conversion to acetaminophen,[20] some evidence suggests that phenacetin *per se* is also active as an analgesic and antipyretic agent[21] and intrinsic analgesic-antipyretic activity of phenacetin has been noted in rats on the basis of the observation that inhibitors, such as SKF 525 A, of liver microsomal enzymes actually increase the potency of the drug.[21b]

The plasma concentrations of phenobarbital were not decreased 90 min after administration of the combined analgesic as compared with those after 45 min in both groups, indicating that the elimination of the drug from plasma is extremely slow. This is also supported by the finding that the elimination of phenobarbital from plasma after intravenous administration was very slow; the elimination rate constant was 0.00204 min^{-1}.[22] Our results suggest that phenobarbital hardly enhances its own metabolism after 7 days' treatment, whereas this drug significantly increases the metabolism of other drugs administered simultaneously.

Ship and Kourounakis[19b] suggested that the effect of an inducer on liver microsomal enzymes is neither increased nor decreased by the presence of a second, less potent inducer. Simultaneous administration of maximal doses of the carcinogens 3-methylcholanthrene and benzpyrene does not result in stimulation of enzyme activity beyond that found for either compound alone[23] and the same result has been observed for phenobarbital and chlordane.[24] Our experiments also showed that the induction rate of drug-metabolizing enzymes with this combined analgesic was almost the same as that obtained with phenobarbital alone, although aminopyrine is also an inducer (Table III). Of these three drugs, phenobarbital may initially combine with the receptor site in the initial step of the induction process. It is known that each drug induces the same type of P-450. These are type I components,[25] whereas acetaminophen is a type II compound.[26]

The decreased analgesic action of the combined analgesic after repeated treatment may be a complicated phenomenon which includes other factors besides induction of hepatic drug-metabolizing enzymes. For example, it has been reported that phenobarbital pretreatment enhances hepatic blood flow[27] and that the hepatic removal rate of oxyphenbutazone depends directly upon the hepatic blood flow.[28] The phenobarbital-induced increase in hepatic blood flow was found to be proportional to liver size, so that blood flow per gram of liver in the pretreated rats was unchanged.[27b] The increased liver size after repeated dosing in the present study thus may suggest an enhancement of the hepatic blood flow and somewhat increased hepatic extraction, although no significant difference in size between single and repeated treatment was found.

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The decreased analgesic action observed after repeated dosing is ascribed largely to metabolism in the liver, but the contribution of the intestinal mucosa must also be taken into consideration, because oxidative biotransformation of foreign compounds by intestinal mucosa or microsomes prepared from the mucosa has been demonstrated in several species.[29] Intestinal microsomes from the rat were found to exhibit a relatively high activity in hydroxylating aromatic hydrocarbons, though aniline hydroxylase and ethylmorphine demethylase were not detected.[29b]

In conclusion, the decreased analgesic action of the combined analgesic after repeated dosing seems to be mainly related to increased metabolism of the components. The facts that the analgesic activity of this combined analgesic decreased markedly after repeated treatment and that the liver drug-metabolizing enzyme activities were enhanced significantly and to a similar extent by the combined drug and phenobarbital lead us to suggest that phenobarbital in the combined analgesic induced an increased level of metabolic inactivation of other component drugs, while aminopyrine and phenacetic in the combined drug did not have such an effect. These findings are clearly of importance in multiple drug therapy.

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