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Comparative N-Demethylation of Antihistaminic Agents possessing Dimethylaminoalkyl Group by Liver Preparations from Four Animal Species

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The N-demethylation of five antihistaminics possessing diemethylaminoalkyl group was compared in *in vitro* production of formaldehyde by liver 9000 g supernatant fraction from four animal species, rat, mouse, guinea pig and rabbit. Sex difference was observed in only rats but the demethylating activities varied from species to species for a drug and from drug to drug in a species. The high demethylating activities were shown for trimeprazine or diphenhydramine by rats and for fenethazine or chlorpheniramine by rabbits. In adult male rats, castration or SKF 525A reduced the activities and 19-nortestosterone phenylpropionate enhanced the activities without the big change in relative order. Phenobarbital induced the activities of female rat liver in almost same proportion for every drug but 3-methylcholanthrene did not show stimulatory effect. The formaldehyde production from N-oxides was comparatively small and did not show the difference among drugs. No correlation between the lipid solubility and the N-demethylation was found. The significance of investigation of species differences in the drug metabolism was discussed.

The species difference in the drug metabolism is an important factor in the mechanism of drug action.[2] It is difficult to predict the metabolism and action of a drug in one species from the data in another species. In addition, the rate of metabolism in a species also varies from drug to drug even though their chemical structures are very similar. Although a number of investigations have been done concerning the relationship between chemical structure and pharmacological activity, it should reconsider this relationship from the metabolic point of view. This communication deals with the comparison of N-demethylation of five antihistaminic agents possessing dimethylaminoalkyl group by liver preparations from four animal species.

Material and Method

Chemicals——The following drugs are gift from the manufacturing company respectively: diphenhydramine hydrochloride (Kowa Co.), chlorpheniramine maleate and 19-nortestosterone phenylpropionate (Sankyo Co.), trimeprazine tartrate (Daiichi-Seiyaku Co.), fenethazine hydrochloride and isothipendyl hydrochloride (Sumitomo Kagaku Co.), and SKF 525A (Smith Kline and French Co.). The chemical structures of five antihistaminics examined are shown in Chart 1. The N-oxides of antihistaminics were synthesized by the usual methods[3] for N, N-dimethylaminoalkyl derivatives but N,S-dioxides were obtained for phenothiazine derivatives. The physicochemical data of these oxides are shown in Table I. Glucose-6-phosphate (G-6-P), G-6-P dehydrogenase and NADP were purchased from Böhringer Co. Other chemicals were of reagent grade and purchased from commercial sources.

Animals——Wistar-King rats, ddN mice, guinea pigs and white rabbits were used and maintained on Clea rat or rabbit chow and water *ad libtum*.

Pretreatment of Animals——Phenobarbital (50 mg/kg) dissolved in distilled water containing 1.1 equivalent NaOH or 3-methylcholanthrene (20 mg/kg) dissolved in corn oil was injected subcutaneously once a

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2) H.B. Hucker, *Ann. Rev. Pharmacol.*, **10**, 99 (1970).
3) A.C. Cope and E. Ciganek, "Organic Syntheses," Coll. Vol. IV, ed. by N. Rabjohn, John Wiley and Sons, Inc., New York. N.Y., 1963, p. 612.

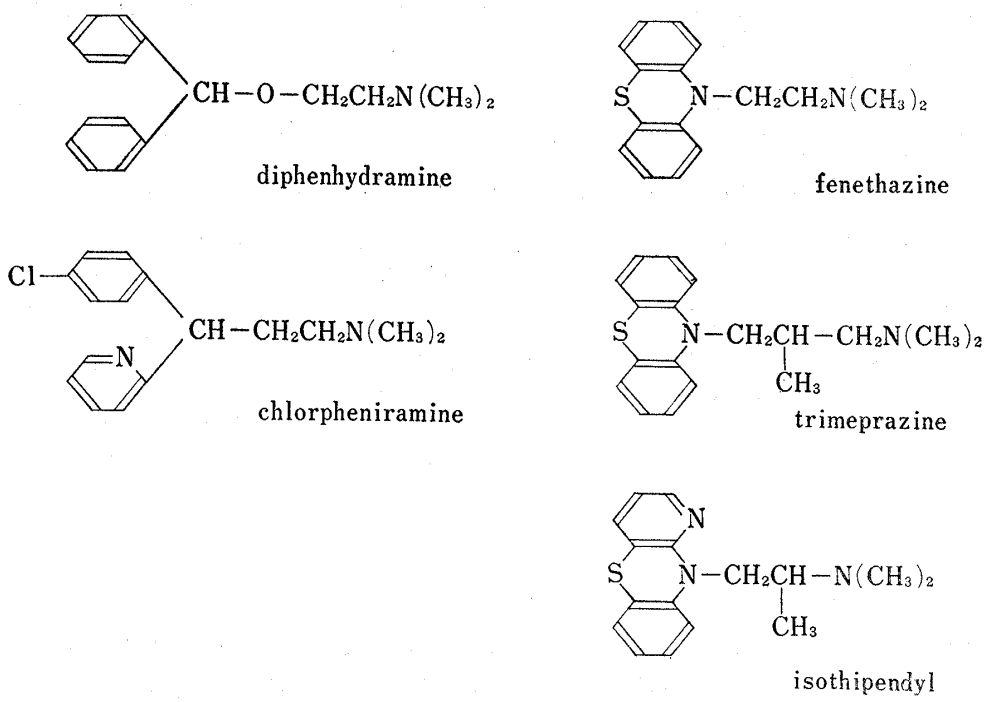

Chart 1. Chemical Structure of Antihistaminic Agents Used in the Experiment

TABLE I. Physicochemical Properties of N-Oxides of Antihistaminics

Oxides[a]	mp[b] (°C) (decomp.)	IR (KBr) cm^{-1} N→O	IR (KBr) cm^{-1} S→O	UV λ_{max}^{EtOH} mμ (ε)	NMR[c] ppm $N(CH_3)_2$ ↓ O
Diphenhydramine N-oxide	100—101	853.0	—	—	3.56(s)
Chlorpheniramine N-oxide	120—121	853.5	—	263.0 (4139)	3.50(s)
Fenethazine N,S-dioxide	156—158	852.0	1020	271.0 (19850) 295.0 (28640) 340.0 (32445)	3.60(s)
Trimeprazine N, S-dioxide	143—145	853.0	1010	234.5 (5667) 272.0 (8516) 296.5 (12670) 343.0 (16090)	3.10(d)
Isothipendyl N, S-dioxide	128—130	852.5	1013 1033	276.5 (16710) 337.0 (32445)	3.58(s)

a) All are hydrogen maleate and recrystallized from i-PrOH and ether.
b) uncorrected
c) Measured in $CDCl_3$ containing tetramethylsilane as internal reference at 60Mc. s: singlet, d: doublet. NMR spectra of parent tertiary amine show 2.78 (d), 2.80 (s), 2.82 (d), 2.48 (s) and 2.80 (s), respectively.

day for three days. SKF 525A (25 mg/kg) dissolved in saline was injected intraperitoneally 40 min before sacrifice. Castration was performed 4 weeks before experiment and 19-nortestosterone phenylpropionate (0.5 mg/kg) in oil solution was injected subcutaneously once a day for 6 days in last week.

Assay of Antihistaminic Activity——An aliquot of incubation mixture of male rat liver 9000 g supernatant with chlorpheniramine was diluted with Tyrode solution and added into a Magnus bath setting a strip of guinea pig ileum. The solution of histamine HCl was added to 5×10^{-6}M and the contraction was determined.

Assay of N-Demethylation by Liver 9000 g Supernatant——The animals were sacrificed by decapitation or strike on the head. After perfusing with ice cold saline, the livers were homogenized with 2 volumes of 0.1 M phosphate buffer, pH 7.4.

An incubation mixture consisted of 2 ml of 9000 g supernatant (667 mg of liver), 5 μmoles of substrate, 0.5 μmoles of NADP, 10 μmoles of G-6-P, 50 μmoles of $MgCl_2$, 100 μmoles of semicarbazide HCl, 100 μmoles of

nicotinamide and the total volume was made to 5 ml with 0.1 M phosphate buffer, pH 7.4. The incubation was carried out at 37° for 30 min. The amount of formaldehyde formed was determined by the method of McFadyen, *et al.*[4]

Assay of N-Demethylation by Liver Microsomes——The microsomal pellets prepared by the usual centrifugation procedure from 9000 g supernatant were suspended in 1.15% KCl. The total volume of an incubation mixture was 3 ml and the constituents were 1 ml of microsomal suspension (0.5 or 1 g of liver), 0.5 ml of 300 mM Tris-HCl buffer, pH 7.4, 15 μmoles of $MgCl_2$, 7.2 μmoles of G-6-P, 1 μmole of NADP, 0.14 units (10 μl) of G-6-P dehydrogenase, and substrate. For the comparison of parent drugs with their N-oxides, the concentration of substrate was 0.5 mM. For the kinetic analyses, five different concentrations over a range of 0.025 to 0.8 mM of substrates were examined. Formaldehyde was determined by Nash[5] and protein by Lowry, *et al*[6].

Lipid Solubility——The solution (1×10^{-3} M) of an antihistaminic in 0.1 M phosphate buffer, pH 7.4, was shaken with an equal volume of *n*-heptane for 20 min. The optical density of aqueous solution at a maximum wave length in ultraviolet (UV) range was determined before (B) and after (A) shaking and heptane/water (H/W) partition coefficient was calculated as H/W = (B -A)/A. The H/W values for diphenhydramine, chlorpheniramine, fenethazine, trimeprazine and isothipendyl were 23, 3, 134, 303 and 45, respectively.

Result

N-Demethylation and Antihistaminic Activity

The typical data (Table II) show that the antihistaminic activity of chlorpheniramine decreased corresponding to the increase of the production of formaldehyde in course of incubation time. This fact suggests that the N-demethylation of antihistaminics might play a definitive role for the disappearance of pharmacological activity.

Table II. N-Demethylation and Antihistaminic Activity of Chlorpheniramine Incubated with 9000 g Supernatant Fraction from Male Rat Liver

Addition	N-Demethylation H CHO formed μmole/g	Antihistaminic activity Contraction Inhibition	
		mm	%
None		75	
Diluted incubation mixture at			
0 min	0	11	85.3
15	0.59	29	61.3
30	1.17	56	25.3
60	1.30	73	2.7

Final concentration of both chlorpheniramine and histamine was 5×10^{-6}M.

Species and Sex Differences in N-Demethylation

Table III presents the comparison of N-demethylating activities for five antihistaminics by liver 9000g supernatant fraction from four animal species. As shown in part A of this table, there are remarkable differences among either species or drugs, but the sex differences were recognized only in rats.

Rats showed the high activities for trimeprazine and diphenhydramine. Mice have high activities for trimeprazine but weak for diphenhydramine. The activities of guinea pig liver were generally low but the demethylation of fenethazine was highest in this species. In rabbits, the very high activities were observed for fenethazine and chlorpheniramine but low for trimeprazine.

4) D.A. McFadyen, H.D. Watkins and P. R. Anderson, *J. Biol. Chem.*, **158**, 107 (1945).
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Part B of Table III presents the inter-drug relative activities to the isothipendyl demethylating activity in each animal group, because the enzymatic activity for this substrate was lowest in three species except guinea pig and the species difference was relatively small. These

Table III. Species and Sex Differences in N-Demethylation of Antihistaminics by Liver 9000 g Supernatant Fraction

	Rat		Mouse		Guinea pig		Rabbit
	Male	Female	Male	Female	Male	Female	Male
Body weight, g	100—120		10—15		450—500		2000
No. of animals[a]	8	5	25	25	3	3	2
A. HCHO formed, μmoles/g liver/hr							
Diphenhydramine	3.50±0.06	2.73±0.09[b]	1.75±0.21	1.85±0.21	1.66±0.29	1.50±0.27	2.68, 2.92
Chlorpheniramine	2.69±0.11	2.30±0.15[d]	2.37±0.26	2.54±0.29	0.86±0.10	0.99±0.13	4.53, 5.87
Fenethazine	2.13±0.10	1.39±0.09[b]	2.14±0.20	2.20±0.25	2.39±0.39	2.46±0.25	6.07, 7.20
Trimeprazine	4.01±0.17	3.07±0.18[b]	2.70±0.12	2.75±0.19	2.18±0.52	2.50±0.45	2.29, 2.23
Isothipendyl	1.85±0.09	1.26±0.03[b]	1.45±0.07	1.63±0.21	1.99±0.41	2.15±0.34	1.64, 1.92
B.[d] Inter-drug relative activities in each animal group, %, (isothipendyl=100)							
Diphenhydramine	192±10	217± 4	119± 9	114±2	85± 5	69± 2	163, 152
Chlorpheniramine	147± 9	181± 9[c]	162±11	156±2	48±11	46± 2	276, 254
Fenethazine	115± 4	111± 6	147±11	136±4	122± 7	117± 9	370, 375
Trimeprazine	217± 8	245±12	187± 7	173±4	109± 4	118±14	140, 116

All values are mean±standard error excepting rabbits.
a) Each liver preparation from one animal was determined for five substrates except that the livers of 5 mice were pooled for one determination.
b) Statistical analyses for the difference between sexes, $p<0.01$.
c) Statistical analyses for the bifference between sexss, $p<0.05$.
d) The values were calculated from individual determinations.

Table IV. Age Difference and Effects of Castration, SKF 525A and Anabolic Steroid Pretreatment on N-Demethylation of Antihistaminics by Liver 9000 g Supernatant Fraction of Male Rats

	Immature	Younger adult	Older adult			
			Normal		Castrated	
			Control	SKF 525A	Control	19-Nor-testosterone
Body weight, g	50	100—120	200—250			
No. of rats[a]	3	8	7	4	2	4
A. HCHO formed, μmoles/g liver/hr						
Diphenhydramine	1.61±0.12[b]	3.50±0.06	4.96±0.31[b,c]	2.26±0.51[d]	1.71	5.62±0.39
Chlorpheniramine	0.62±0.28[b]	2.69±0.11	3.19±0.43	1.24±0.25[c]	1.24	3.58±0.25
Fenethazine	0.66±0.20[b]	2.13±0.10	3.44±0.26[b]	1.55±0.25[c]	0.72	4.24±0.09
Trimeprazine	1.06±0.32[b]	4.01±0.17	4.48±0.48	1.71±0.57[c]	1.41	5.25±0.38
Isothipendyl	0.69±0.22[b]	1.85±0.09	3.44±0.44[b]	1.58±0.37[c]	1.29	3.96±0.17
B.[d] Inter-drug relative activities in each animal group, % (isothipendyl=100)						
Diphenhydramine	476±64[e]	192±10	152±31[e]	143± 7	133	144±16
Chlorpheniramine	124±16	147± 9	91±11[b]	83±11	96	91± 6
Fenethazine	97± 4	115± 4	104± 7	101± 4	56	110± 6
Trimeprazine	154± 7	217± 8	134±11[b]	111± 1	109	134±14

Values are mean±standard error except only mean for castrated control group.
a) Each liver preparation from one rat was determined for five substrates.
b) Significantly different from younger adult, $p<0.01$.
c) Significantly different from older adult, $p<0.01$.
d) The values were calculated from individual determinations.
e) Significantly different from younger adult control, $p<0.05$.

relative activities were not significantly different between sexes in any species except that the difference in chlorpheniramine by rat liver was slightly significant.

Age Differences and Effects of Castration SKF, 525A or 19-Nortestosterone in Male Rats

Table IV presents the N-demethylating activities of male immature (50—60 g), younger adult (100—120 g), and older adult (200—250 g) rats. The N-demethylating activities became higher with the growth but the difference among drugs became smaller. The activity for diphenhydramine was about 2.5 fold of that for isothipendyl in immature rats but only 1.5 fold in older adults.

The pretreatment with SKF 525A or castration reduced the activities and the administration of anabolic steroid, 19-nortestosterone phenylpropionate, restored the activities of castrated rats toward or over normal level.

Effects of Phenobarbital and 3-Methylcholanthrene in Female Rats

The pretreatment with phenobarbital markedly enhanced the activities of N-demethylation of every drug in almost same proportion. No stimulatory action was observed by 3-methylcholanthrene. These data are shown in Table V.

Table V. Effects of Phenobarbital and 3-Methylcholanthrene on N-Demethylation of Antihistaminics by Liver 9000g Supernatant of Female Rats

	Control	Phenobarbital	3-Methyl-cholanthrene
No. of rats (70 g)[a]	6	5	5
A. HCHO formed μmoles/g liver/hr			
Diphenhydramine	2.26±0.28	6.87±0.36[b]	2.51±0.25
Chlorpheniramine	1.71±0.31	4.34±0.54[b]	1.74±0.18
Fenethazine	0.79±0.13	3.76±0.27[b]	0.84±0.10
Trimeprazine	1.87±0.44	6.87±0.53[b]	2.04±0.27
Isothipendyl	0.78±0.09	2.36±0.14[b]	0.73±0.05
B.[d] Inter-drug relative activities, % (isothipendyl=100)			
Diphenhydramine	309±12	295±25	345±31
Chlorpheniramine	216±21	184±23	239±25
Fenethazine	100±11	161±14[d]	116±13
Trimeprazine	230±29	295±30	274±20

All values are mean±standard error.
a) Each liver preparation from one rat was determined for five substrates.
b) Significantly different from control, $p < 0.01$.
c) The values were calculated from individual determinations.
d) Significantly different from control, $p < 0.05$.

Comparison of N-Demethylation

Table VI summarizes the order of N-demethylating activities with statistical analyses for difference between adjacent in each animal group. In rats, the activities for trimeprazine and diphenhydramine are higher than those for other three. The difference between trimeprazine and diphenhydramine is not so much and the rough classification, T-D and F-C-I, is not changed, although the order in T-D or F-C-I is changed by treatment or age. However, the difference between trimeprazine and diphenhydramine is greater in mice or guinea pigs than in rats.

N-Demethylation from N-Oxide Derivatives

The N-demethylation from N-oxide derivatives were compared with those from tertiary amine type drugs using liver microsomes from male rats. As shown in Table VII, the formaldehyde production from N-oxides was very smaller than that from corresponding parent drugs.

Table VI. Comparative Summary of N-Demethylation

Species	Sex[a]	Body weight g	Treatment	Order and difference[b]
Rat	M	50	No[c]	D, T, F, I, C
		100	No	T>D>>C>>F>>I
		200	No	D=T>F=I=C
		200	SKF 525 A	D>T>I=F=C
		200	castration[c]	D, T, I, C, F
		200	castration and anabolic steroid	D=T=F=I=C D>F, T=I, T>>C, F>C
	F	100	No	T>D>>C>>F=I
		70	No	D=T=C>F=I, D>>C
		70	phenobarbital	D=T>C=F>>I
		70	3-methylcholanthrene	D=T=C>F=I
Mouse	M, F	10—15	No	T=C>F>>D>>I
Guinea pig	M, F	450—500	No	F=T=I>>D>>C
Rabbit	M	2000	No[c]	F, C, D, T, I

a) M: Male, F: Female
b) T: Trimeprazine, D: Diphenhydramine, C: Chlorpheniramine, F: Fenethazine, I: Isothipendyl
>: $p < 0.05$, >>: $p < 0.01$, =: $p > 0.05$, non-significant
c) Not statistically analyzed because of small sample.

Table VII. Formation of Formaldehyde from Antihistaminics and their N-Oxides by Liver Microsomes of Male Rats

	HCHO formed, mμmole/mg protein/min	
	from parent drug	from N-oxide
Diphenhydramine	2.21	0.39
Chlorpheniramine	1.45	0.45
Fenethazine	1.16	0.37
Trimeprazine	1.89	0.39
Isothipendyl	1.22	0.48

Livers from 5 rats weighing about 80 g were combined.

Kinetic Constants for the N-Demethylation by Liver Microsomes from Male Rats and Rabbits

The most remarkable difference among drugs and species was for fenethazine or trimeprazine in rats or rabbits as shown in Table III. Table VIII presents the kinetic constants for N-demethylation by liver microsomes from these species. The differences in *K*m values

Table VIII. Kinetic Constants for the N-Demethylation of Antihistaminics by Liver Microsomes from Male Rats and Rabbits

	K_m 10^{-4} M		V_{max} mμmole/mg protein/min	
	Rat	Rabbit	Rat	Rabbit
Chlorpheniramine	1.5	0.9	3.47	5.56
Fenethazine	1.0	2.0	3.17	8.00
Trimeprazine	1.7	0.9	6.13	2.46
Isothipendyl	1.0	1.0	2.76	2.33

These values represent typical experiments which have been repeated two or three times. Each value was obtained from pooling tissues from 5 rats or from the liver of one rabbit.

are not so much but Vmax for fenethazine in rabbit and Vmax for trimeprazine in rat were quite high reflecting to the high activities in 9000g supernatant fraction.

Discussion

It is obvious that there are great differences in the N-demethylating activities of liver microsomal fraction for antihistaminics possessing dimethylaminoalkyl group in spite of their resemble chemical structure and pharmacological action. The sex differences were observed only in rats as well known, but the activity for each drug varies from species to species. For example, trimeprazine is highly demethylated by rat liver but relatively low by rabbit liver and the relationship between species and substrate for fenethazine is reverse.

According to Liberman,[7] rabbits tolerated doses up to 100 mg/kg of fenethazine with little effects but rats showed respiratory difficulties at 20 mg/kg and spasms at 30 mg/kg. On the contrary, when the same dose of trimeprazine (100 mg/kg) were given, little effect was observed in rats but rabbit lost the response to stimulation for over 24 hr (our preliminary experiment). Since the N-demethylation results in the disappearance of pharmacological report or toxicity as supposed from the experiment with chlorpheniramine (Table II) or the action on cyclizine or chlorcyclizine,[8] these phenomena are interpreted by the rapid demethylation of fenethazine in rabbit and that of trimeprazine in rat.

McMahon[9] proposed that the lipid solubility of a substrate was rate-limiting factor for liver microsomal demethylation. Standing the results obtained by rat or mouse microsomes for a variety of substrates, Mazel and Henderson[10] doubted this relationship. McMahon[11] further pointed out that the effect of any single parameter would be obscured in a group of randomly selected compounds which were not chemically related. However, in spite of close similarity of our substrates, the rate of demethylation completely reversed in species. If the lipid solubility would be rate-limiting factor, the relative activities among drugs or the order of activities should be same in any species.

The lipid solubility of trimeprazine was high and its demethylation by rat was also high but that by rabbit was low. The solubility of chlorpheniramine was lowest among five substrates but its demethylation was not always lowest. The solubility of isothipendyl was not so small but its demethylation was usually low. It was impossible to correlate the lipid solubility of drugs with the N-demethylating activities in any species.

There is the question as to whether N-oxides are intermediary compounds in the mechanism of N-demethylation of dimethylaniline.[12] No parallelism was observed between the production of formaldehyde from parent drugs and that from their N-oxides. It is unlikely that N-oxides involve so much in the substrate differences in N-demethylation of antihistaminics.

The existence of more than one system for the demethylation has been discussed from the kinetic evidence,[13] but it was unable to conclude from our data. However, it was recognized that Vmax were more reflected to the activities of 9000g supernatant fraction of liver.

In general, the species differences in the metabolism of drugs occur in both the rate and the direction.[2] Other metabolic pathways such as aromatic hydroxylation, sulfoxidation, etc., must be considered, because these pathways might have some effects on the N-demethylation. Furthermore, even if the rate of demethylation does not obey the lipid solubility theory, the

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overall metabolic rate including other pathways might obey this theory. It should be emphasized that the metabolism of drugs has to be investigated in the well controlled conditions of animals for various pathways.

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