COMPARATIVE TOXICOKINETIC STUDY OF RUBBER ANTIOXIDANTS, 2-MERCAPTOBENZIMIDAZOLE AND 2-MERCAPTOMETHYLBENZIMIDAZOLE, BY SINGLE ORAL ADMINISTRATION IN RATS

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ABSTRACT — Toxicokinetics of 2-mercaptobenzimidazole (MBI) and 2-mercaptomethylbenzimidazole (MMBI), rubber antioxidants with thioureylene structure, were compared after single oral administration in rats. Male Wistar rats received single oral administration of 2, 10, 50 and 250 mg/kg of MBI or MMBI. The serum and urine concentrations of MBI and MMBI were determined by HPLC. MBI and MMBI showed similar C_{max} values, but the former disappeared slower in the serum than the latter and resulted in its larger AUC values. Analyses of MBI, MMBI and their desulfurated metabolites in urine suggested that these differences were due to their metabolic elimination rates. On the other hand, MBI and MMBI caused similar acute toxicities, such as loss of locomotive activity, ataxic gait, adoption of prone or side position and coma, being severer with higher serum concentrations at the moment. Similar acute toxicities between MBI and MMBI were explained by similar C_{max} values at the same dose. It was suggested from these results that the slower disappearance and larger AUC values of MBI in the serum compared to MMBI might explain the strong thyroid toxicity which has been observed by repeated administration of MBI, but very weak thyroid toxicity by MMBI.

KEY WORDS: 2-Mercaptobenzimidazole, 2-Mercaptomethylbenzimidazole, Toxicokinetics, Acute toxicity, Rubber antioxidant, Rats

INTRODUCTION

2-Mercaptobenzimidazole (MBI) and 2-mercaptomethylbenzimidazole (MMBI) are rubber antioxidants with thioureylene structure (Fig. 1). MMBI is a methyl derivative of MBI and has been supplied as a 1:1 mixture of 4-methyl and 5-methyl isomers (Isama *et al.*, 1998). Single oral administration toxicity studies of MBI and MMBI showed that they have similar acute toxicity. Both MBI and MMBI caused reduced spontaneous activity, paralytic gait, lacrimation and adoption of the prone position in rats by single oral administration, and their LD_{50} values are reported to be 300 and 330 mg/kg, respectively (Kawasaki *et al.*, 1998; Saitoh *et al.*, 1999).

Repeated administration toxicity studies, however, revealed the markedly different potency of thyroid toxicity

between MBI and MMBI, although they share the responsible thioureylene structure. It has been shown that oral administration of MBI causes thyroid enlargement, decreased thyroxine and triiodothyronine, and an increased thyroid-stimulating hormone in rats (Janssen *et al.*, 1981; Gaworski *et al.*, 1991; Kawasaki *et al.*, 1998). In a 28-day repeated oral administration toxicity study in rats, MBI caused a two- to three-fold increase in thyroid weight with clear histopathological changes at 10 mg/kg/day (Kawasaki *et al.*, 1998). On the other hand, 100 mg/kg/day of MMBI caused only a 1.8-fold increase in thyroid weight with moderate histopathological changes in spite of the same study protocol (Saitoh *et al.*, 1999).

These differences in thyroid toxicity between MBI and MMBI may be due to their toxicokinetics and toxicodynamics (TK/TD). However, available data for their

TK/TD are limited. A disposition study using radio-labeled MBI showed that about 40-60% and 70% were excreted in 24 hr and 72 hr urine, respectively, when 0.5 or 49 mg/kg was orally administered to rats (El Dareer *et al.*, 1984). Benzimidazole (BI) has been identified as one of the major metabolites of MBI in rats (Janssen *et al.*, 1981, El Dareer *et al.*, 1984). No data are available for the toxicokinetics of MMBI. In the present study, we established an analytical method for TK study and compared serum concentration profiles in rats after single oral administrations of MBI or MMBI. Urinary excretions of MBI, MMBI and their desulfurated metabolites were also compared.

MATERIALS AND METHODS

Test chemicals

2-Mercaptobenzimidazole (MBI, CAS No. 583-39-1, RTECS No. DE1050000, M.W. 150.2) and benzimidazole (BI, CAS No. 51-17-2) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). 2-Mercaptomethylbenzimidazole (MMBI, CAS No. 53988-10-6, Nocrac MMB) was supplied from Ohuchi Shinko Chemical Ind., Ltd. (Tokyo, Japan). 2-Mercapto-5-methylbenzimidazole (5-MMBI, M.W. 164.2, CAS No. 27231-36-3) and 5-methylbenzimidazole (5-MeBI, M.W. 132.2, CAS No. 614-97-1) were purchased from Aldrich Japan Inc. (Tokyo, Japan). 2-Mercapto-4-methylbenzimidazole (4-MMBI, CAS No. 27231-33-0) was isolated from MMBI by repeated fractional recrystallization (Isama *et al.*, 1998).

Experimental animals

Specific-pathogen-free Wistar male rats (4-weeks old) were purchased from SLC Co. (Shizuoka, Japan) and acclimatized for one week prior to use. The basal pellet diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were given *ad libitum*. The animal room was maintained at a temperature of $24 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ humidity with a 12-hr light/dark cycle. For observation of acute toxicity, three to five rats were kept in plastic cages with chip bedding. For the collection of urine, rats were kept individually in a metabolic cage made of stainless steel.

Administration of test chemicals

MBI and MMBI were dissolved at low concentrations or were suspended at high concentrations in corn oil to make the dose volume at 5 ml/kg. Rats were fasted from 16 hr before administration until 24 hr after administration. For oral administration, MBI or MMBI

was given to six rats per dose by gavage at a dose of 0 (control), 2, 10, 50 or 250 mg/kg. For intraperitoneal (i.p.) administration, MBI or MMBI was given to two rats per dose by i.p. injection at a dose of 0 (control) or 50 mg/kg.

Observation of acute toxicity

Toxic signs of rats were observed at the time of blood collection. After the termination of blood and urine collections, rats were sacrificed by bleeding under ether anesthesia and autopsied. Rats that died during the course of the experiment were autopsied as soon as possible.

Collections of blood and urine

Blood was collected from the orbital plexus of the rats under ether anesthesia at the designated times after administrations: 0.5, 1, 2, 4, 6, 10, 24, 34 and 48 hr for the oral MBI, 0.5, 1, 2, 4, 6, 10 and 24 hr for the oral MMBI, and 0.5, 2, 4, 10 and 24 hr for the i.p. MBI and MMBI. In order to reduce the frequency of blood collection per animal, the staggered method (Dahlem *et al.*, 1995) was employed; i.e., in each of the dose groups, six rats/group were divided into two subgroups ($n = 3$) and blood was collected alternately. Serum was prepared from the blood and stored at -20°C until analysis.

Urine was individually collected for 24 hr after the oral administration of MBI or MMBI from one of the subgroups in each dose group under fasted conditions. After the addition of 10% volume of 1N NaOH as a bacteriostat, the urine was stored at -20°C until analysis.

Fig. 1. Chemical structures of MBI, MMBI and their metabolites. Thioureylene structure is depicted in bold face.

Analysis of MBI, MMBI and their metabolites

Serum was deproteinized by the addition of two volumes of methanol followed by centrifugation at 12,000 rpm for 5 min. The obtained supernatant was diluted with the mobile phase and analyzed by HPLC (Model LC-4A, Shimadzu, Kyoto, Japan). The column was μ Bondapak C18 ODS (3.9 mm ϕ $\times$ 300 mm, Waters, MA, USA) and the mobile phase was 0.1% phosphoric acid: methanol 1:3 (v/v). HPLC was operated at a column oven temperature of 45°C with a flow rate at 0.5 ml/min and with a detection wavelength at 300 nm. The quantification limits for MBI and MMBI were 0.08 μ g/ml in serum.

Urine was diluted with two volumes of distilled water and centrifuged at 12,000 rpm for 5 min. The supernatant obtained was analyzed by HPLC (Model LC-10A equipped with a photodiode array UV-VIS detector, Model SPD-M6A, Shimadzu, Kyoto, Japan). The column was CAPCELL PAK C18 ODS (4.6 mm ϕ $\times$ 250 mm, Shiseido, Tokyo, Japan) and the mobile phase was acetonitrile: 0.01M phosphate buffer (pH 7.0) 1:9 (v/v). The operating conditions of HPLC were the same as for serum analysis. The quantification limits for MBI and MMBI were 0.075 μ g/ml in urine. Major metabolite of 4-MMBI in urine detected at 271 nm was collected and analyzed by mass spectrometry (JMS-AM II, JEOL, Tokyo, Japan).

Calculation of Toxicokinetic Parameters

Toxicokinetic parameters were obtained by non-compartmental analysis using a commercially available computer program (Win Nonlin-Standard Version, Ver. 1.1, Scientific Consulting, Inc., North Carolina). AUC₀₋₂₄ was calculated by the trapezoidal rule. C_{max} and T_{max} were based on the highest observed concentration.

Statistical analysis

Data were expressed as the mean $\pm$ S.D.. Statistical evaluation of the data was not performed.

RESULTS

Acute toxicity of MBI and MMBI

No toxic signs were observed by single oral administrations of MBI or MMBI at 50 mg/kg or less. At 250 mg/kg, both MBI and MMBI caused the decrease in locomotor activity, ataxic and paralytic gait, prone or side position, lacrimation, gasping and coma. Five out of six rats orally given 250 mg/kg of MBI died from 12 hr to 50 hr after treatment and one rat survived. Five out of six rats orally given 250 mg/kg of MMBI died

within 26 hr after treatment and one rat survived. At autopsy, there were no toxicological findings at any dose of MBI. Apparent enlargement of the liver with a faded color was observed at 250 mg/kg of MMBI. Single i.p. administration of MBI or MMBI at 50 mg/kg caused the decrease in locomotor activity from 30 min for 3-4 hr.

Serum concentration profiles of MBI and MMBI

The serum concentration-time profile of MBI is indicated in Fig. 2A. The serum concentrations of MBI at early hr were proportional to the dose but unproportional in AUC, indicating non-linear kinetics. MBI in serum disappeared slowly and was detected over 48 hr at the higher doses. The C_{max} of MBI after i.p. administration at 50 mg/kg was 2.3-fold higher than that after oral administration of the same dose, suggesting that absorption of MBI from the abdominal cavity was rapid and the first path effect less significant in i.p. route administration.

The serum concentration-time profile of MMBI is indicated in Fig. 2B. The serum concentrations of MMBI were similar to those of MBI at early hr, although undetected at 2 mg/kg. Furthermore, the serum concentration of MMBI after i.p. administration suggested rapid transfer from the abdominal cavity and a less significant first path effect compared to MBI. However, MMBI in serum disappeared faster than MBI, being undetectable even at 12 hr at 50 mg/kg. When 4-MMBI and 5-MMBI were determined separately after oral administration of MMBI at 50 mg/kg, the former disappeared slower than the latter, indicating the apparent effect of the position of methyl substituent on the toxicokinetics of MMBI (Fig. 2C).

Toxicokinetic parameters of MBI and MMBI

The toxicokinetic parameters of MBI and MMBI are shown in Table 1. The proportional C_{max}, delayed T_{max} and unproportionally high AUC of MBI after oral administration were observed at the higher doses, suggesting that the absorption and/or elimination rate of MBI is lower at the higher doses. Similar tendencies were also observed for MMBI, but to a lesser extent. The differences of AUC values between MBI and MMBI were smaller at higher doses; e.g., the AUC₀₋₂₄ of MBI was only 2.5-fold larger than that of MMBI at 250 mg/kg, but as large as 9.9-fold at 10 mg/kg. On the other hand, the differences of C_{max} values between MBI and MMBI were relatively small at all doses, i.e., with C_{max} of MBI 1.3- to 1.7-fold greater than that of MMBI. These results suggest an accelerated elimina-

Fig. 2. Serum concentration profiles of MBI and MMBI after single oral administration.

A) Serum concentration-time profiles of MBI after single oral and i.p. administrations to rats.

B) Serum concentration-time profiles of MMBI after single oral and i.p. administrations to rats.

C) Serum concentration-time profiles of 4-MMBI and 5-MMBI after single oral administration of 50 mg/kg MMBI to rats.

Blood samples were collected by the staggered method as described in Materials and Methods. Closed symbols show mean values of three rats/subgroup with vertical bars indicating S.D.. Open circles show values from one or two rats.

N.D.: Lower than quantification limit.

*1: Average of two rats.

*2: Average of two rats survived until 48 hr in the two subgroups.

*3: Average of five rats survived until 24 hr in the two subgroups.

tion rate of MMBI compared to MBI.

Relationship of serum concentration with toxic signs of MBI and MMBI

Individual serum concentrations symbolized with toxic signs at blood collection time, which depict serum concentrations in identical animals at different hr, are plotted against dose in Fig. 3. For both MBI and MMBI, higher serum concentrations were accompanied by severer toxic signs, and low serum concentrations showed no or weak toxic signs, such as decrease in locomotor activity, even at the highest administration dose. It is noteworthy that i.p. administration of both MBI and MMBI at 50 mg/kg showed an apparent decrease in locomotor activity, but oral administration at the same dose did not show any toxic signs consistent with their serum levels. It is thus considered that for both MBI and MMBI, the expression of toxic signs depends on the serum concentration of the moment, irrespective of the dose administered.

Urinary excretion of MBI and MMBI

MBI, 4- and 5-MMBI in urine were identified by HPLC analysis. As desulfurated metabolites of MBI and 5-MMBI, BI and 5-MeBI (Fig. 1), respectively, were detected at 271 nm and identified by HPLC equipped with a photodiode array UV-VIS detector using authentic substances (Fig. 4A). As a desulfurated metabolite of 4-MMBI, 4-MeBI was identified by mass spectrometry analysis of a presumed peak fraction that was collected by preparative HPLC at 271 nm (Fig. 4B). The mass spectrum of the presumed 4-MeBI fraction shared fragments of m/z 132 $[M]^+$ with authentic 5-MeBI (data not shown).

The amounts of MBI and MMBI with their desulfurated metabolites excreted in urine for 24 hr after oral administration are shown in Fig. 5. The amount of MBI in urine was roughly proportional to the dose administered. On the other hand, the amount of BI in urine was unproportionally low at 250 mg/kg and was almost the same as 50 mg/kg, suggesting metabolic saturation of the desulfuration of MBI. The sum amounts of 4- and 5-MMBI were apparently lower at the doses of less than 50 mg/kg but similar at 250 mg/kg compared to MBI. Conversely, the amounts of 4- and 5-MeBI were higher than BI at the same doses. Metabolic saturation for desulfuration was also suggested for 4- and 5-MMBI as for MBI, since the amounts of 4- and 5-MeBI were similar or higher at 50 mg/kg than at 250 mg/kg. These results suggest that the desulfuration capacity of MBI and MMBI is limited, and is more

efficient for the latter than the former. In addition, it is suggested that the urinary excretion rates of MBI and MMBI are similar under conditions of saturated desulfuration capacity.

DISCUSSION

The present results indicate the difference in the toxicokinetics between MBI and MMBI after single

oral administration at the same doses. The plot of serum concentration versus toxic signs in individual animals showed that the acute toxicities of MBI and MMBI depend on serum concentration at the moment (Fig. 3). This explains the similarity in the acute toxicities between MBI and MMBI, which have been considered to indicate effects on the central nervous system (Kawasaki *et al.*, 1998; Saitoh *et al.*, 1999). Although MBI disappears much slower and has larger AUC val-

Fig. 3. Plot of serum concentration versus toxic signs of MBI and MMBI after single oral and intraperitoneal administrations. Each symbol means the toxic sign observed at the time of blood collection.

Table 1. Toxicokinetic parameters of 2-mercaptobenzimidazole (MBI) and 2-mercaptomethylbenzimidazole (MMBI) in male rats after single oral and intraperitoneal administrations.

Compounds	Dose (mg/kg)	C _{max} (µg/ml)	T _{max} (hr)	AUC ₀₋₂₄ (µg·hr/ml)
MBI	2	0.2 ± 0.04	0.5	0.84
	10	3.2 ± 0.37	1	35.6
	50	14.9 ± 0.60	6	278.1
	50 (i.p.) ^a	34.4 ± 0.08	0.5	474.5
	250	65.4 ± 17.30	10	2529.9
MMBI	2	N.D.	N.D.	N.D.
	10	1.9 ± 0.26	0.5	3.61
	50	9.8 ± 1.85	0.5	72.9
	50 (i.p.) ^b	25.2	2	142.4
	250	52.3 ± 2.22	2	1009.4

Results of oral administrations are expressed as mean ± S. D. of three rats for C_{max}.

AUCs were calculated from mean values of three rats (but serum concentration data for 34 and 48 hr of 250 mg/kg of MBI were averages of two rats).

N.D.: lower than quantification limit.

^a: Data from two rats.

^b: Data from one rat.

Fig. 4. HPLC chromatograms of authentic samples of MBI and MMBI, and their desulfurated metabolites monitored by 271 nm.

A: Authentic sample containing 25 μg/ml of each compound.

B: Urine sample from the rat receiving 50 mg/kg of MMBI.

Fig. 5. Urinary excretion of MBI, MMBI and their desulfurated metabolites during 24 hr after single oral administration to rats.

A: Urine from the three rats receiving MBI.

B: Urine from the three rats receiving MMBI.

*1 : Urine from two rats, because one rat went into coma and did not excrete urine during 24 hr.

ues in the serum than MMBI, they have similar C_{max} values at the same administration doses and therefore cause similar acute toxicities. These findings are also consistent with the notion that the drugs acting on the central nervous system show effects associated with their blood concentration (Wright *et al.*, 1993). Thus, the present results are good examples of the significance of toxicokinetic data in individual animals as mentioned in the guidance for toxicokinetic study by ICH (1994).

Although their C_{max} values were similar, the slower disappearance of MBI in the serum resulted in larger AUC values for MBI than for MMBI. These differences of AUC are considered to be mainly due to their metabolic elimination rates. This is because the differences of AUC values between MBI and MMBI are smaller at 250 mg/kg dose, when the urinary excretion rates of MBI and MMBI are similar, probably due to saturation of the metabolic process including desulfuration.

In conclusion, MBI in the serum disappeared much slower than MMBI and resulted in larger AUC after single oral administration, indicating that systemic exposure level is greater for MBI than for MMBI at the same dose. By repeated administration, the difference of systemic exposure level might become larger than that by single administration due to contributing factors such as metabolic enzyme induction. This may provide an explanation for their markedly different thyroid toxicity exhibited by repeated administration, although our recent *in vitro* study of MBI and MMBI on inhibitory activity (as IC₅₀) of lactoperoxidase revealed it to be 20.6 and 43.7 μ M, respectively, showing a comparable antithyroid potential of both thioureylene compounds (Sakemi and Tsuda, unpublished data.). To verify this explanation, a study on toxicokinetics by repeated administration and further *in vitro* study on toxicodynamics for MBI and MMBI are in progress in our laboratory.

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