Stereoselective Reductive Metabolism of Metyrapone and Inhibitory Activity of Metyrapone Metabolites, Metyrapol Enantiomers, on Steroid 11β-Hydroxylase in the Rat

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Pharmacokinetics of metyrapone and metyrapol enantiomers was studied in the rat to determine the stereoselective reductive metabolism of metyrapone. The HPLC method using a chiral column was developed for the stereoselective analysis of metyrapol enantiomers in rat plasma. The *AUC* ratio of (−)- and (+)-metyrapol appeared in rat plasma after i.v. administration of metyrapone was about 3:1. The interconversion of (−)- or (+)-metyrapol to its antipode was negligible, and the reverse reaction from metyrapol to metyrapone was insignificant. There were similar kinetic parameters of (−)-metyrapol to those of (+)-metyrapol after i.v. administration of racemic metyrapol. These results indicate metyrapone displays product-stereoselective reductive metabolism in the rat. The inhibition of steroid 11β-hydroxylase by metyrapone, racemic metyrapol, (−)-metyrapol or (+)-metyrapol was analyzed in rat adrenal homogenates. Metyrapol was equally as potent as metyrapone in the inhibition of steroid 11β-hydroxylase and each enantiomer of metyrapol showed similar inhibitory activity on the rat adrenal steroid 11β-hydroxylase. These results indicate there is an insignificant difference in the inhibitory effects on steroid 11β-hydroxylase of metyrapol enantiomers, and that the inhibitory effects of metyrapol may be involved in the pharmacological activity of metyrapone *in vivo*.

Key words metyrapone; enantiometric metabolite; stereoselective reduction; pharmacokinetics; rat adrenal; 11β-hydroxylase

Numerous carbonyl containing drugs undergo carbonyl reduction as a major biotransformation step and the corresponding alcohol is formed. These drugs are excreted mainly in the urine as glucuronides. We have investigated the alcohol–ketone conversion and pharmacokinetics of 4-substituted acetophenone derivatives such as acetohexamide.[1-3] These drugs and the metabolites formed by reduction of the aromatic keto group undergo reversible biotransformation.

Metyrapone (2-methyl-1,2-bis-(3-pyridyl)-1-propanone; MO; Fig. 1) is a well-known inhibitor of adrenal steroid 11β-hydroxylase in man and animals, and is used clinically as a diagnostic test agent for pituitary–adrenal function.[4] However, the reversible transformation between MO and its aromatic reductive chiral metabolite, metyrapol (2-methyl-1-[3-(6-oxopyridyl)]-2-(3-pyridyl)-1-propanone; MOH), has not been investigated. The major metabolic pathways of MO in rat liver have been reported to be keto reduction[5] and pyridine N-oxidation.[6] The reduction of a ketone group of MO forms an asymmetric carbon in the molecule of MOH. Recently, Chiarotto and Wainer reported the determination of MO and MOH enantiomers simultaneously using coupled achiral-chiral liquid chromatography.[7] MOH was also reported to be an inhibitor of steroid 11β-hydroxylase as potent as MO.[8-10] However, little information about the stereoselective inhibitory activity of MOH enantiomers has been reported.

In this study we developed an analytical method of MOH enantiomers in rat plasma by HPLC using a chiral column, and we examined whether the metabolic reaction from MO to MOH in the rat displays product stereoselectivity. The pharmacokinetics of (−)-MOH and (+)-MOH in the rat were also investigated after i.v. administration of MOH. We also examined whether there is a stereoselective difference in the inhibitory effect of MOH enantiomers on steroid 11β-hydroxylase using rat adrenal homogenates.

MATERIALS AND METHODS

Chemicals MO and deoxycorticosterone (DOC) were obtained from Sigma Chemical Co. (St. Louis, MO). Corticosterone was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo). Progesterone was obtained from Steraloids Inc. (Wilton, NH). Phenacetin was obtained from Hishiyama Pharmaceutical Co., Ltd. (Osaka). β-Nicotinamide adenine dinucleotide phosphate ($NADP^+$) was obtained from Oriental Yeast Co., Ltd. (Tokyo). Glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase (G-6-P dehydrogenase) were obtained from Boehringer Mannheim (Mannheim). (±)-MOH was prepared by reducing MO with $NaBH_4$ in methanol and its mp was 99—101 °C (from ethyl acetate) and $[\alpha]_D = \pm 0°$ ($c = 1.0$, ethanol). The enantiomers (−)-MOH and (+)-MOH were purified by preparative liquid chromatography (PLC) with a Chiralpak AD column (250 × 10 mm i.d., Daicel Chemical Ind., Tokyo) from racemic MOH. The optical rotation of (−)-MOH was $[\alpha]_D = -29.0°$ ($c = 0.70$,

Metyrapone (MO) Metyrapol (MOH)

Fig. 1. Chemical Structure of MO and MOH
* Chiral carbon.

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ethanol) and that of (+)-MOH was $[\alpha]_D = +27.1°$ (c = 0.69, ethanol). The optical purity of both enantiomers was over 98.0%. All other chemicals were of analytical reagent grade.

Chromatographic Conditions The HPLC-system was combined with a M600 multi-solvent delivery system and a 990J photodiode array detector (Waters, Milford, MA). (−)-MOH and (+)-MOH were analyzed on a Chiralpak AD column (250 × 4.6 mm i.d., Daicel Chemical Ind., Tokyo). The mobile phase was *n*-hexane–ethanol (7 : 3, v/v) at a flow rate of 1.0 ml/min, and the absorbance was detected at a wavelength of 265 nm.

The peak of MO overlapped with the peak of solvent under the chromatographic conditions described above. To analyze MO, the HPLC-system was combined with a LC-6A delivery system and a SPD-6A UV detector (Shimadzu, Kyoto) with a Cosmosil $5C_{18}$ column (150 × 4.6 mm i.d., Nacalai Tesque Inc., Kyoto). The mobile phase was acetonitrile–water (3 : 7, v/v) at a flow rate of 1.0 ml/min, and the absorbance was detected at a wavelength of 250 nm at ambient temperature.

Corticosterone and DOC were analyzed on a Cosmosil $5C_{18}$ column (250 × 4.6 mm i.d., Nacalai Tesque Inc.). The mobile phase was methanol–water (7 : 3, v/v) at a flow rate of 1.0 ml/min, and the absorbance was detected at a wavelength of 245 nm.

Animals and Drug Administrations Male Wistar rats (Japan SLC Inc., Shizuoka), weighing 200—250 g, were anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneally). MO or (±)-MOH dissolved in phosphate buffer pH 7.4 was administered intravenously at a dose of 0.1, 0.25 or 0.5 mmol/kg. Thereafter, blood samples (500 μl) were collected through a cannula inserted into the jugular vein after various periods, then centrifuged immediately to separate the plasma, which was stored at 4 °C until assay.

Extraction Procedure A plasma sample (200 μl) was added to 6 ml of chloroform containing phenacetin as the internal standard (0.54 μg/ml), and made up to 8 ml with distilled water. The mixture was shaken and centrifuged for 10 min at 3000 rpm. After centrifugation, the organic phase (5 ml) was evaporated *in vacuo*, then the residue was dissolved in 100 μl of ethanol and 20 μl was analyzed on a Chiralpak AD column or Cosmosil $5C_{18}$ column. The recoveries of MO and MOH extracted from rat plasma were over 98.0%.

Reaction mixture (1 ml) of *in vitro* sample was stopped by adding 1 ml of acetonitrile and was extracted using 6 ml of chloroform containing progesterone as the internal standard (2 μM). The mixture was shaken and centrifuged for 10 min at 3000 rpm. After centrifugation, the organic phase (5 ml) was evaporated *in vacuo*, then the residue was dissolved in 100 μl of ethanol and 20 μl was analyzed on a Cosmosil $5C_{18}$ column.

Figure 2A shows a chromatogram of MO and MOH extracted from plasma at 50 min after the i.v. administration of MO in the rat. The retention times of MOH, phenacetin (I.S.; internal standard) and MO were 4.8, 6.8 and 8.2 min, respectively. The calibration curve of MO showed a good linearity in the range of 0.02—8 mM ($r^2 > 0.99$). Figure 2B shows a chromatogram of the enantiomeric metabolites, (−)-MOH and (+)-MOH, extracted from plasma at 50 min after the i.v. administration of MO to the rat. Phenacetin (I.S.), (−)-MOH and (+)-MOH were eluted with retention times of 5.8, 8.8 and 12.6 min, respectively. The calibration curves obtained by plotting the peak area ratio of each compound in the range of 0.01—5.0 mM to I.S. showed good linearity ($r^2 > 0.99$).

Pharmacokinetics Analysis Plasma concentration data after a single i.v. injection of MO fit a two-compartment open model. Model parameters were calculated using the program MULTI.[11] Plasma clearance (CL) was obtained by $Dose/(A_1/\lambda_1 + A_2/\lambda_2)$. Volume of distribution at steady state (V_{ss}) was obtained by $V_1(1 + k_{12}/k_{21})$. The formation clearance, $CL_{MO \to (-)\text{-MOH}}$ or $CL_{MO \to (+)\text{-MOH}}$, is the proportion of the conversion from MO to the corresponding enantiomeric metabolite, (−)-MOH or (+)-MOH. $CL_{(-)\text{-MOH}}$ or $CL_{(+)\text{-MOH}}$ is the plasma clearance of (−)-

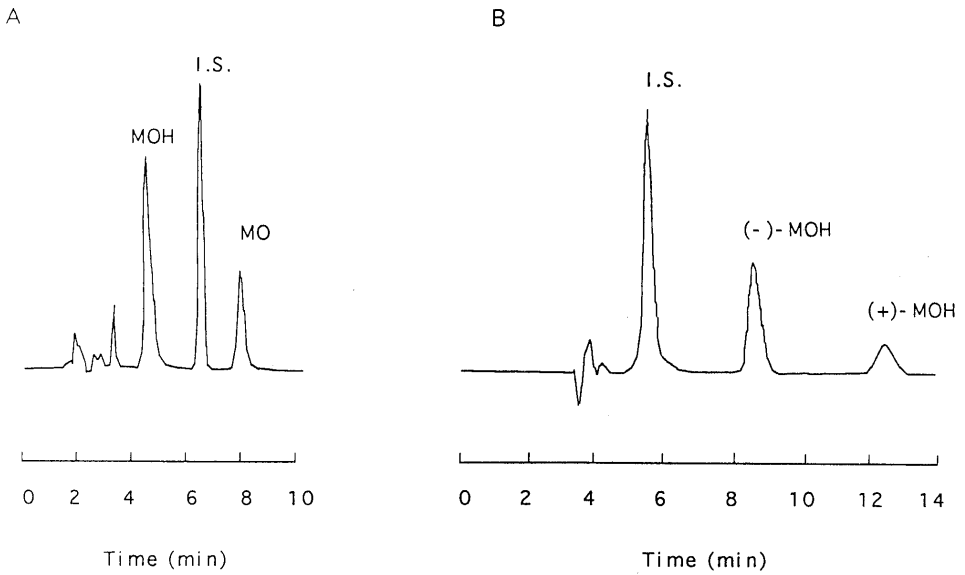

Fig. 2. Chromatograms of the Extract from Rat Plasma 50 min after the i.v. Administration of 0.25 mmol/kg of MO

Conditions for HPLC were as given in the Materials and Methods. (A) Chromatogram with Cosmosil $5C_{18}$ column; (B) chromatogram with Chiralpak AD column.

MOH or (+)-MOH, respectively, after the administration of racemic MOH. $AUC^{MO}_{(-)\text{-MOH}}$ and $AUC^{MO}_{(+)\text{-MOH}}$ are the total areas under the concentration–time profiles of (−)-MOH and (+)-MOH after the administration of MO. AUC_{MO} and AUC_{MOH} are the total areas under the drug and metabolite concentration–time profiles, respectively. The formation clearance was obtained by the following equation[12]:

$$CL_{MO\rightarrow(-)\text{- or }(+)\text{-MOH}} = CL_{(-)\text{- or }(+)\text{-MOH}} \cdot AUC^{MO}_{(-)\text{- or }(+)\text{-MOH}} / AUC_{MO}$$

A preliminary experiment was carried out to determine the partition coefficient for metyrapone between blood cell and plasma. The blood sample with the final concentration of MO, 15 μg/ml was incubated for 60 min at 37 °C and whole blood and plasma concentration was determined by HPLC. The blood cell to plasma concentration ratio of MO was about 0.8 after correction of the haematocrit.

Preparation of Adrenal Homogenates Rats were fasted overnight prior to experiments, but drinking water was allowed *ad libitum*. Animals were decapitated and adrenals were homogenized in 3 volumes of 0.01 M phosphate buffer (pH 7.4) containing 1.15% (w/v) KCl with a Potter-Elvehjem Teflon-glass homogenizer.

The protein concentration was determined by the Bio-Rad protein assay[13] using bovine serum albumin as the standard.

Enzyme Assays The inhibitory potencies on the steroid 11β-hydroxylase of MOH enantiomers were compared using rat adrenal homogenates. DOC was used as a substrate. Incubation mixture consisted of phosphate buffer (0.1M, pH 7.4), an NADPH generating system ($NADP^+$ (0.2 μmol), G-6-P (1 μmol), G-6-P dehydrogenase (0.2 units)), $MgCl_2$ (5 μmol), adrenal homogenates (finally about 0.3 mg protein/ml), the inhibitor (final concentration 0.4 mM) and 0.2 mM DOC dissolved in ethanol to give a final volume of 1.0 ml.[5,8,14] After 10 min preincubation, the reaction was started by adding the substrate and the mixture was incubated at 37 °C for 5 min in adrenal homogenates. The time course of the production of corticosterone in adrenal homogenates showed a linearity over an incubation period of 5 min. The reaction was stopped by adding 1 ml of acetonitrile to the mixture, and the product was extracted by adding 6 ml of chloroform containing internal standard and determined by HPLC. The retention time of corticosterone, DOC and progesterone (I.S.) was 7.8, 10.0 and 13.0 min, respectively.

RESULTS

Pharmacokinetics of MO and MOH in Rats Plasma concentration–time curves of MO, (−)-MOH and (+)-MOH after the i.v. administration of 0.25 mmol/kg dose of MO are shown in Fig. 3. The plasma concentrations of (−)-MOH and (+)-MOH formed by metabolism exceeded MO concentration at 45 and 90 min, respectively, after the administration. The pharmacokinetic parameters of MO, (−)-MOH and (+)-MOH after the i.v. administration of 0.1, 0.25 and 0.5 mmol/kg dose of MO are summarized in Table 1. The *CL* and V_{ss} of MO were about 0.6 l/h·kg and 0.7 l/kg, respectively. The *AUC* ratio of (−)-MOH to (+)-MOH was about 3 : 1 in the range of doses from 0.1 to 0.5 mmol/kg.

Plasma concentration–time curves of (−)-MOH and (+)-MOH, after the i.v. administration of 0.1, 0.25 and 0.5 mmol/kg dose of racemic MOH, are presented in Fig. 4. The pharmacokinetic parameters of (−)-MOH and (+)-MOH are summarized in Table 2. The *AUC* value varied in proportion to the administration dose in the range of 0.1—0.5 mmol/kg and the difference of *AUC* values between MOH enantiomers in this dose range was insignificant. After racemic MOH administration, the *AUC* ratio of (−)-MOH to (+)-MOH was about 1 : 1.

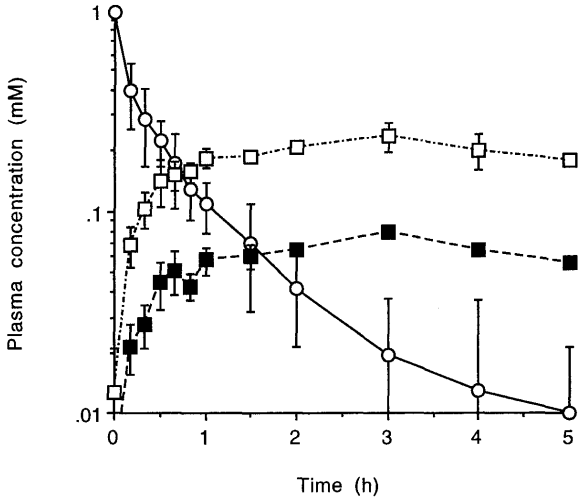

Fig. 3. Plasma Concentration of the Metabolic Isomers of MOH and MO after i.v. Administration of MO

○, MO after administration of a 0.25 mmol/kg dose; □, (−)-MOH and ■, (+)-MOH formed after a 0.25 mmol/kg dose of MO. Each point represents the mean ± S.D. (n = 3 or 4).

Table 1. Pharmacokinetic Parameters[a] Calculated after i.v. Administration of MO

Administered drug	Dose (mmol/kg)	Analyzed compound	Parameter			Analyzed compound	Parameter
			AUC (mM·h)	*CL* (l/h·kg)	V_{ss} (l/kg)		AUC_{1-5h} (mM·h)
MO	0.1	MO	0.18±0.07	0.57±0.07	0.78±0.18	(−)-MOH	0.29±0.02[c] (2.90)[b]
						(+)-MOH	0.10±0.04
	0.25	MO	0.44±0.18	0.53±0.18	0.80±0.40	(−)-MOH	0.77±0.30[c] (2.65)
						(+)-MOH	0.29±0.01
	0.5	MO	0.87±0.26	0.60±0.13	0.70±0.18	(−)-MOH	1.77±0.13[c] (2.46)
						(+)-MOH	0.72±0.05

a) Each value indicates the mean ± S.D. of 3—5 experiments. *b*) Parentheses indicate the *AUC* ratio of (−)-MOH to (+)-MOH. *c*) $p<0.05$, significantly different from the corresponding *AUC* value of (+)-MOH.

Table 2. Pharmacokinetic Parameters[a] of (−)-MOH and (+)-MOH after i.v. Administration of Racemic-MOH in Rats

Administered drug	Dose (mmol/kg)	Analyzed compound	Parameter		
			AUC (mM·h)	CL (l/h·kg)	V_{ss} (l/kg)
Racemic-MOH	0.1 (0.05)	(−)-MOH	0.50±0.03 (1.19)[b]	0.10±0.01	0.63±0.15
	(0.05)	(+)-MOH	0.42±0.03	0.11±0.01	0.74±0.15
	0.25 (0.125)	(−)-MOH	1.41±0.33 (1.27)	0.09±0.01	0.64±0.10
	(0.125)	(+)-MOH	1.11±0.04	0.11±0.01	0.62±0.10
	0.5 (0.25)	(−)-MOH	2.78±0.66 (1.01)	0.09±0.02	0.53±0.06
	(0.25)	(+)-MOH	2.75±0.40	0.09±0.01	0.55±0.05

a) Each value indicates the mean±S.D. of 3—5 experiments. b) Parentheses indicate the AUC ratio of (−)-MOH to (+)-MOH.

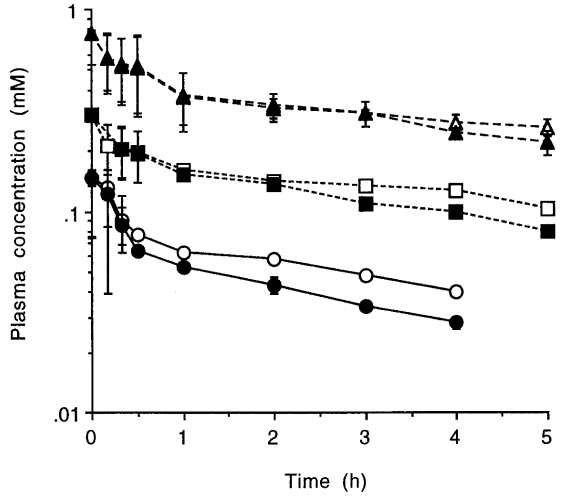

Fig. 4. Plasma Concentration of the Optical Isomers Appearing after the i.v. Administration of Racemic MOH

△, (−)-MOH and ▲, (+)-MOH after a 0.5 mmol/kg dose; □, (−)-MOH and ■, (+)-MOH after a 0.25 mmol/kg dose; ○, (−)-MOH and ●, (+)-MOH after a 0.1 mmol/kg dose of MOH. Each point represents the mean±S.D. (n=3 or 4).

Both enantiomers had similar pharmacokinetic parameters of AUC, CL and V_{ss} after racemic MOH administration. After the administration of (±)-MOH, MO formation in rat plasma was insignificant, indicating that MO and MOH did not undergo reversible transformation in the rat.

The metabolism of MO *in vitro* was investigated using rat liver 10000 ***g*** supernatant. The incubation mixture consisted of an NADPH generating system, $MgCl_2$, rat liver 10000 ***g*** supernatant and MO (2 mM). The formation ratio of (−)-MOH to (+)-MOH was about 3:1. This *in vitro* result was consistent with that obtained by *in vivo* study. When (−)- or (+)-MOH (2 mM) was incubated for 3 h in the same manner as MO, concentration of (−)- or (+)-MOH throughout the 3-h incubation period showed little change and the formation of its antipode was insignificant. It was assumed that the chiral inversion between (−)-MOH and (+)-MOH did not occur in the rat.

Thus, the clearance of each MOH enantiomer was similar in the rat in the range of doses studied. Urinary recovery of MO by 24 h after i.v. administration was negligible.

The formation clearance to (−)-MOH or (+)-MOH from MO was calculated as described in the Materials and Methods (Table 3). The formation clearance of (−)-MOH from MO was 3 times larger than that of (+)-MOH.

Inhibitory Effects on Steroid 11β-Hydroxylase Activity of MO, (−)-MOH, (+)-MOH or (±)-MOH MO, (−)-MOH, (+)-MOH or (±)-MOH was added to incubation mixtures as an inhibitor and the inhibitory effects on steroid 11β-hydroxylase activity were obtained by measuring corticosterone formation from DOC in rat adrenal homogenates. The inhibitory effects in these homogenates were 39.7% in the presence of MO, 40.8% in (±)-MOH, 33.8% in (−)-MOH, and 38.1% in (+)-MOH (Table 4). This indicated that there was insignificant difference in the inhibitory effects for 11β-hydroxylase of (−)-MOH and (+)-MOH and the potency of MOH was the same as MO in rat adrenal tissue.

Table 3. Formation Clearance[a] of (−)-MOH and (+)-MOH after i.v. Administration of MO

Administered drug	Dose (mmol/kg)	$CL_{MO\to(-)\text{-MOH}}$ (l/h·kg)	$CL_{MO\to(+)\text{-MOH}}$ (l/h·kg)
MO	0.1	0.36±0.06 (3.00)[b]	0.12±0.02
	0.25	0.38±0.18 (2.92)	0.13±0.04
	0.5	0.38±0.08 (2.71)	0.14±0.02

a) Each value indicates the mean±S.D. of 3—5 experiments. b) Parentheses indicate the formation clearance ratio of (−)-MOH to (+)-MOH.

Table 4. Inhibitory Effects of MO, (−)-MOH and (+)-MOH on 11β-Hydroxylase in Rat Adrenal Homogenates

Inhibitor	Activity[a] (nmol/min/mg protein)	Inhibition (%)
None	16.33±2.37	—
MO	9.86±0.44	39.7
MOH	9.68±1.26	40.8
(−)-MOH	10.81±2.69	33.8
(+)-MOH	10.11±1.18	38.1

a) Each value indicates the mean±S.D. of 6 experiments. Concentration of inhibitor: 0.4 mM.

DISCUSSION

We developed a determination method of MOH enantiomers in rat plasma using an amylose derivative type chiral column. The value of enantioselective resolution was 2.12, higher than the previously described value (1.06) by Chiarotto and Wainer.[7]

There are few reported studies on the product stereoselectivity of MO reductive metabolism. In this paper, the pharmacokinetics of MO and its metabolites formed by keto reduction, (−)-MOH and (+)-MOH, after i.v.

administration of MO was studied in male Sprague-Dawley rat. Damani's group[15,16] reported pharmacokinetics of MO and MOH in the rat. According to their data[16] the blood clearance and volume of distribution at steady state after i.v. administration of MO (50 mg/kg) were estimated to be 1.21 l/h·g and 1.22 l/kg. Their clearance data of MO is about 2 times larger than our present data. Presumably, this difference in clearance can be attributed to the difference in strain of rats or difference in the experimental conditions such as anesthesia or collected sample, because we carried out the experiments using a plasma sample under pentobarbital anesthesia, but they examined using a whole blood sample under an unanesthetized condition.[15,16]

MOH enantiomers showed a slower clearance than MO after i.v. administration of MO. The *AUC* ratio of (−)-MOH to (+)-MOH was about 3 : 1 in the range of doses from 0.1 to 0.5 mmol/kg. After i.v. administration of racemic MOH the kinetic behavior of each MOH enantiomer gave similar values and no interconversion between (−)-MOH and (+)-MOH took place in the rat. The ratio of the formation clearance of (−)-MOH from MO to its (+)-MOH from MO was about 3 : 1, showing the product stereoselectivity in the keto-reduction of MO in the rat. Chiarotto and Wainer[7] reported that the existence of the product stereoselectivity in the keto-reduction of MO was observed in man, and each enantiomer of MOH showed higher plasma concentration than MO at 2 and 4 h after MO administration. Considered together with our results and the report by Chiarotto and Wainer,[7] MOH may be contribute to the enzyme inhibitory activity of MO in man.

The sum of the formation clearance to (−)-MOH and (+)-MOH from MO corresponded to about 90% of the total clearance of MO. This suggested that most of the disappearance of MO in the rat was due to the reductive metabolism to MOH from MO.

MO is a well-known inhibitor of steroid 11β-hydroxylase.[4] Colby and Brownie[8] indicated that MOH was nearly as effective as MO in rat adrenal cortex for the inhibition of steroid 11β-hydroxylase. According to Sprunt and Hannah,[9] the relative potency for steroid 11β-hydroxylase inhibition of MOH was 70% as potent as MO in rat adrenal tissue and was equally as potent as MO in human adrenal tissue. We examined the effect of MOH enantiomers on steroid 11β-hydroxylase inhibition in rat adrenal homogenates. The inhibitory potencies for the 11β-hydroxylase indicated that each enantiomer of MOH was almost as active as MO.

In conclusion, the existence of product stereoselectivity in the keto-reduction of MO was confirmed. The relative potencies for steroid 11β-hydroxylase inhibition of MOH enantiomers were as active as MO in rat adrenal tissue. In addition, MOH after i.v. administration of MO showed a higher *AUC* and slower clearance than MO in the rat. Therefore, the inhibitory effects for steroid 11β-hydroxylase of MOH may be involved in the pharmacological action of MO *in vivo*.

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