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ORIGINAL RESEARCH

Simultaneous determination of amantadine and rimantadine by HPLC in rat plasma with pre-column derivatization and fluorescence detection for pharmacokinetic studies

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ABSTRACT: We investigated simultaneous high-performance liquid chromatographic (HPLC) determination of amantadine hydrochloride (AMA) and rimantadine hydrochloride (RIM) levels in rat plasma after fluorescent derivatization with *o*-phthalaldehyde and 2-mercaptoethanol. Afterwards, the method was applied to determine their pharmacokinetics. The retention times of AMA and RIM derivatives were 12.6 and 22.2 min and the lower limits of detection were 0.025 and 0.016 μg/mL, respectively. The coefficients of variation for intra- and inter-day assay of AMA and RIM were less than 5.1 and 7.6%, respectively. After i.v. administration of AMA or RIM to rats, the total body clearance and distribution volume at the steady-state of RIM were higher than those of AMA. Bioavailability of AMA and RIM was 34.9 and 37.2%, respectively. When AMA and RIM were p.o. co-administered, the area under the plasma concentration–time curve of RIM was significantly lower than that after RIM alone. On the other hand, pharmacokinetic parameters of AMA did not significantly change. These results indicate that our HPLC assay is simple, rapid, sensitive and reproducible for simultaneously determining AMA and RIM concentrations in rat plasma and is applicable to their pharmacokinetic studies. Also, co-administration of AMA and RIM may result in the lack of pharmacological effects of RIM. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: amantadine; rimantadine; fluorescent derivatization with *o*-phthalaldehyde and 2-mercaptoethanol; pharmacokinetic study

INTRODUCTION

1-Adamantanamine hydrochloride (amantadine, AMA) has been clinically used as an antiparkinsonism agent as well as an antiviral drug (Bryson, 1982; Paci *et al.*, 2001). For its pharmacokinetic studies, the determination of the levels has been performed by a gas–liquid chromatographic procedure employing flame ionization and electron capture detection in biological fluids (Bleidner WE *et al.*, 1965; Biandrate *et al.*, 1972; Sioufi and Pommier, 1980; Stumph *et al.*, 1980; Belanger and Grech-Belanger, 1982). According to Sioufi and Pommier (1980), the former procedure lacked sensitivity for the reliable determination of plasma levels of AMA. Hesselink *et al.* (1999) investigated the brain penetration of AMA by microdialysis study using gas chromatographic analysis coupled with mass selective detection (GC–MS). [^{3}H]–AMA is frequently utilized for studies on the transport mechanism through the blood–brain barrier and on the reabsorptive mechanism in kidney (Spector, 1988; Wong *et al.*, 1990; Goralski *et al.*, 1999).

1-(1-Adamantyl)ethylamine hydrochloride (rimantadine, RIM), an analog of AMA with reported equal efficacy and fewer adverse reactions than AMA, has been reported to be effective against influenza (Wingfield *et al.*, 1969; Dolin *et al.*, 1982). The pharmacokinetics in humans and transport studies using animal tissues have been investigated mainly using GC–MS and [^{14}C]-RIM (Wills *et al.*, 1987; Hoffman *et al.*, 1988; Spector, 1988; Holazo *et al.*, 1989). In the various experiments described above, complicated equipment and special facilities for using radioactive compounds are necessary. Therefore, a more convenient, sensitive and simple method is required.

The use of a derivatization reagent which is reactive toward the amino group and the more popularized system would seem suitable for determining these

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Abbreviations used: 2-ADA, 2-adamantanamine hydrochloride; BA, bioavailability; AMA, amatidine hydrochloride; 2-ME, 2-mercaptoethanol; OPA, *o*-phthalaldehyde; RIM, rimantadine hydrochloride.

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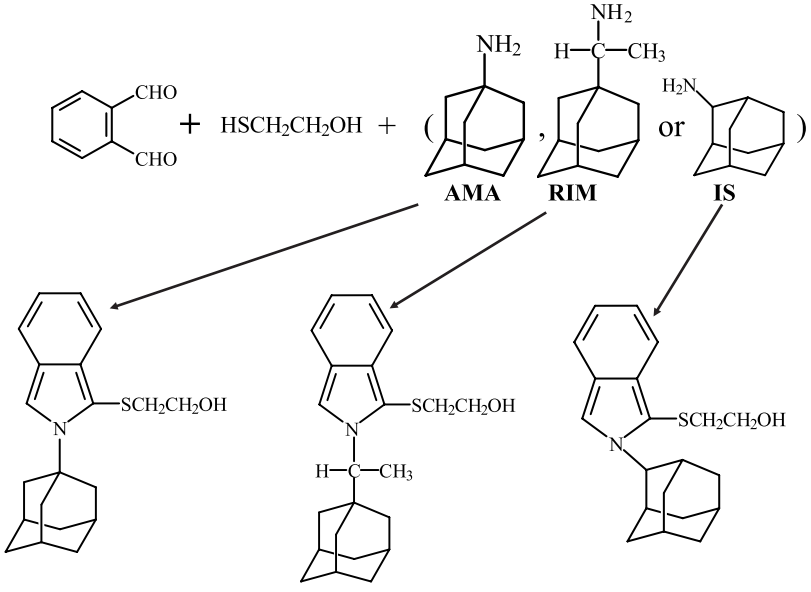

Figure 1. Formation of fluorescent AMA, RIM and IS derivatives.

concentrations in biological fluids. Van der Horst *et al.* (1990) showed the determination of AMA in urine using high-performance liquid chromatography (HPLC) by derivatization with 1-fluoro-2,4-dinitrobenzene as a UV labeled agent. However, the utility resulted in producing an amount of precipitation during the derivatization, and interference peaks were detected on chromatograms. The derivatization using 3-(7′-methoxycoumarin-3′-carbonyl)-benzoxazoline-2-thione was satisfactory with respect to simplicity and precision to quantify AMA spiked in the urine (Fujino *et al.*, 1993). However, the HPLC method has not been widely utilized, because they are not commercially available. Although Desai and Gal (1993) indicated the detection of RIM after pre-column derivatization with *o*-phthalaldehyde (OPA) and *N*-acetyl-L-cysteine, *N*-acetyl-D-penicillamine, 2,3,4,6,-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside or 1-thio-β-D-glucose in borate buffer at pH 9.5, the quantitative assay was not established in the rat plasma sample. Recently, we have shown the simple and quantitative analysis of AMA by HPLC after derivatization with OPA and 1-thio-β-D-glucose using 2-adamantanamine hydrochloride (2-ADA) as an internal standard (IS) in human plasma (Higashi and Fujii, 2004). However, the described thiol agents are too expensive to be clinically utilized. Therefore, it is likely that the development of a procedure using a more inexpensive reagent may be appropriate to promote the quantitative determination of these compounds in biological fluids and be reasonable for therapeutic drug monitoring.

In this study, the quantitative analysis of AMA and RIM by HPLC is investigated after derivatization with OPA and 2-mercaptoethanol (2-ME), which is more than 10–400-fold cheaper per 1 g unit than the described thiol agents, using 2-ADA as an IS in rat plasma according to the reaction shown in Fig. 1. Afterwards, the disposition kinetics of AMA and RIM is investigated in rats using our HPLC method. AMA and RIM pharmacologically possess the same effects, indicating that they may be prescribed in order to increase the effects. However, the effects of RIM and AMA on AMA and RIM disposition kinetics, respectively, have not been examined after co-administration. Moreover, we investigate the pharmacokinetic drug–drug interaction between AMA and RIM in rats.

EXPERIMENTAL

Reagents. AMA, RIM, 2-ADA and OPA were obtained from Aldrich Chemical (Milwaukee, WI, USA). 2-ME, other general reagents and methanol for the HPLC analysis were supplied by Wako Pure Chemical Industries (Osaka, Japan).

Instrumentation and chromatographic conditions. The HPLC system comprised a model L-6200 pump (Hitachi, Tokyo, Japan) and a model RF-10A fluorometer (Shimadzu, Kyoto, Japan) operating at an excitation wavelength of 342 nm and an emission wavelength of 410 nm. The HPLC column (Kanto Chemical, Tokyo, Japan) was 150 × 4.6 mm i.d. and 5 μm particles of C_{18} packing material. Quantification of the peaks was performed with a Chromatopac, model CR-3A integrator (Shimadzu, Kyoto, Japan). The mobile phase was prepared by the addition of methanol (400 mL) to a solution of 100 mL containing acetic acid (0.2 v/v%) in water at pH 7.0 by NaOH. The derivatives were eluted from the column at a flow rate of 0.8 mL/min.

Sample preparation. A 50 μL aliquot of the plasma sample was rendered alkaline by the addition of NaOH (2 M, 200 μL). 2-ADA (1 μg/mL, 50 μL) was added as the IS to show the standard curves of AMA and RIM. Then the mixture was vortexed for 1 min and extracted with freshly distilled *n*-hexane (3 mL, twice). Each *n*-hexane phase was mixed and evaporated, and the derivatization was performed as follows. Derivatization was performed in borate buffer (0.1 M) adjusted to pH 9.5 by the addition of NaOH. A 400 μL aliquot of borate buffer was added to the residue. 2-ME solution (2 v/v% in water, 50 μL) and OPA solution (40 mg/mL in acetonitrile, 50 μL) were added and vortexed. The mixture was allowed to react for 6 min at room temperature and the derivatized sample (25 μL) was injected in the column.

Calibration curves. Solutions of AMA and RIM (1 mg/mL) were prepared in water, stored at 4°C, and then further diluted with rat plasma to the desired concentration (0, 0.01, 0.02, 0.04, 0.1, 0.2, 0.4, 1 and 2 μg/mL) before use. All samples were extracted and analyzed using the procedures described above. Calibration curves based on the peak area ratios of AMA and RIM to IS were analyzed in duplicate for each sample.

Animal experiments. Male Wistar rats (8–10 weeks, 271 ± 20 g; mean ± SD, Sankyo Laboratory Animal, Toyama, Japan) were used in the pharmacokinetic study. Rats received 6.0 and 30 mg/kg (32 and 160 μmol/kg, respectively) doses of AMA or 6.8 and 34 mg/kg (32 and 160 μmol/kg, respectively) doses RIM by the i.v. and p.o. administration, respectively. Under light anesthesia by diethyl ether, blood samples (about 0.2 mL) were withdrawn with heparinized syringes from the carotid vein at designated time intervals (0.033, 0.25, 0.5, 1, 2, 3, 4, 6 and 8 h for i.v. administration and 0.083, 0.25, 0.5, 1, 2, 3, 4, 6 and 8 h for p.o. administration) and collected in tubes. Blood samples collected in the different experiments were centrifuged (1000 ***g***, 5 min) to obtain the plasma. In the same manner, drug-free pooled plasma samples were obtained from rats. The plasma samples were immediately frozen and stored at –18°C until assay.

Pharmacokinetic analysis. The pharmacokinetic parameters of AMA and RIM after i.v. or p.o. administration were estimated by moment analysis. The area under the plasma concentration–time curve from zero to 8 h ($AUC_{0\to 8\,h}$) and the mean residence time after i.v. administration ($MRT_{i.v.}$) were calculated using the linear trapezoidal rule. The absolute bioavailability (BA) after p.o. administration was calculated from mean values of the dose-adjusted $AUC_{0\to 8\,h}$ after p.o. and i.v. administration. Total body clearance (CL_{tot}) and distribution volume at the steady-state (Vd_{ss}) after i.v. administration were estimated as follows: $CL_{tot} = D_{i.v.}/AUC_{i.v.}$, $Vd_{ss} = CL_{tot} \times MRT_{i.v.}$. The peak plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were determined from the actual data obtained after p.o. administration.

Statistical analysis. Data are expressed as the mean ± SD. The statistical significance of the difference between mean values was assessed using the Student's *t*-test. Statistical probability (*p*) values less than 0.05 (two-tailed) were considered significantly different.

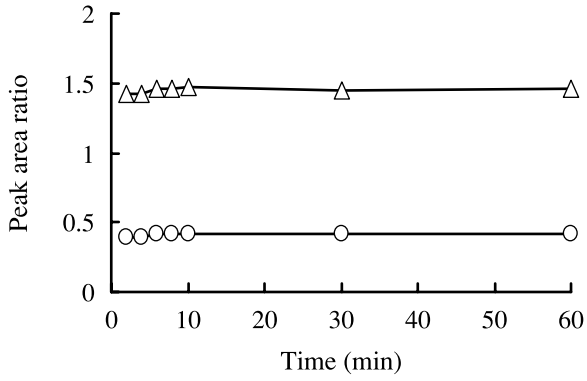

Figure 2. Peak area ratio of AMA (○) and RIM (△) derivatives to IS derivatives as a function of time of reaction.

RESULTS AND DISCUSSION

Reaction time courses

Figure 2 shows the reaction time course of AMA and RIM derivatives with OPA and 2-ME. When AMA and RIM samples (1 μg/mL in rat plasma, 50 μL) containing IS (1 μg/mL in water, 50 μL) were extracted by *n*-hexane and derivatized with OPA and 2-ME at room temperature, the peak area ratio of AMA and RIM derivatives to IS derivative were practically constant after 6 min of reaction. Thus, the derivatization time of 6 min was chosen for the complete reaction in this study.

Chromatograms of AMA and RIM derivatives

Figure 3 shows the chromatograms obtained from (A) drug-free plasma, (B) plasma spiked with AMA, RIM and IS (each 1 μg/mL), (C) plasma at 2 h after p.o. administration of AMA (30 mg/kg) to rats, (D) plasma at 2 h after p.o. administration of RIM (34 mg/kg) to rats and (E) plasma at 2 h after p.o. co-administration of AMA (30 mg/kg) and RIM (34 mg/kg) to rats. Drug-free rat plasma yielded relatively clean chromatograms with no significant interfering peaks. As shown in the chromatogram of (B), the retention times of AMA, RIM and IS derivatives were 12.6, 22.2 and 14.1 min, respectively. This HPLC system is specific for the simultaneous determination of AMA and RIM in rat plasma. The peaks were sharp, indicating high column efficiencies for two derivatives.

Linearity and the limit of detection

The linearity of standard curves of AMA and RIM was displayed for AMA and RIM concentrations ranging from 0.04 to 2 and 0.02 to 2 μg/mL, respectively. These regression coefficients (r^2) were more than 0.991 ($y = 0.4170x - 0.0135$ for AMA; $y = 1.4555x + 0.0088$ for

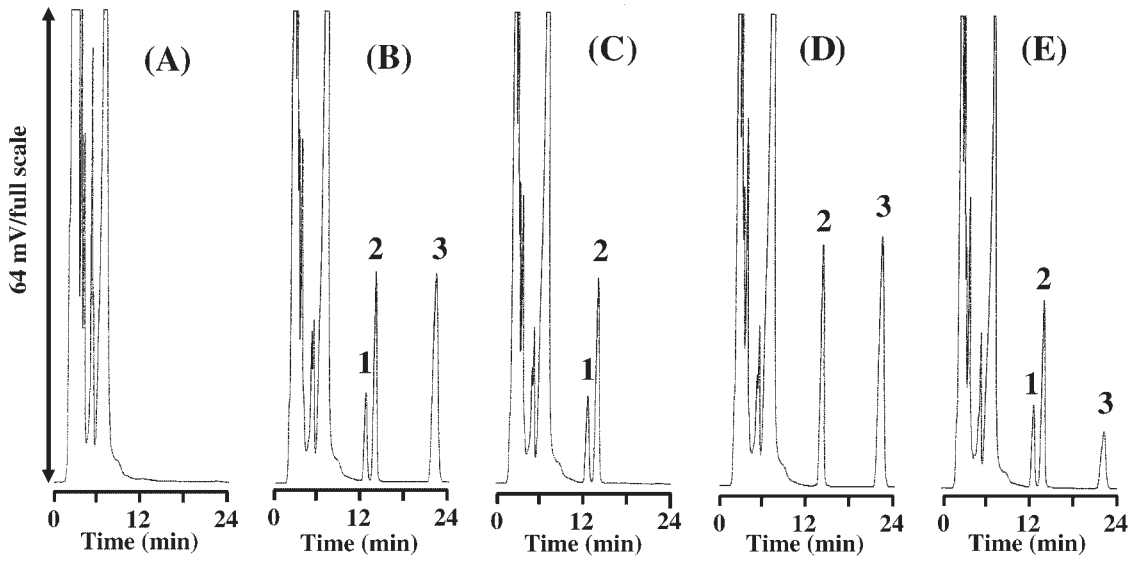

Figure 3. Chromatograms of derivatives with OPA and 2-ME of extracted rat plasma samples. (A) Drug-free plasma; (B) plasma spiked with AMA, RIM and IS (each 1 μg/mL; peaks: **1**, AMA derivative; **2**, IS derivative; **3**, RIM derivative); (C) plasma at 2 h after p.o. administration of AMA (30 mg/kg) to rats; (D) plasma at 2 h after p.o. administration of RIM (34 mg/kg) to rats; and (E) Plasma at 2 h after p.o. co-administration of AMA (30 mg/kg) and RIM (34 mg/kg) to rats. The attenuation for all chromatograms is 64 mV/full scale.

RIM). The lower limits of detection for AMA and RIM utilizing this method were established at 0.025 and 0.016 (signal-to-noise ratio of 3:1), respectively.

Zhou *et al.* (1993) demonstrated the direct determination of AMA in plasma and urine by a solid-phase reagent containing a covalently bound activated ester of 9-fluoreneacetate. While the procedure is very simple, the equipment used is not popular in a clinical setting and the lower limit of quantification of AMA was poor (0.2 μg/mL). Although (2-naphthoxy)acetyl chloride, not commercially available, was recently used as a simple fluorescent reagent, the detection limit of AMA was consistent with our data and a large amount of sample (300 μL) was required (Duh *et al.*, 2003). In a previous pharmacokinetic study after the oral administration of AMA (100 mg) to humans, the plasma levels of AMA were 0.20–1.05 μg/mL (Belanger and Grech-Belanger, 1982). In addition, in rats at an infusion of 3 mg/kg of [^{3}H]-AMA, the range of plasma concentration from 7 to 127 min varied between 0.9 and 0.3 μg/mL (Goralski *et al.*, 1999). While Holazo *et al.* (1989) investigated RIM assay in human plasma by GC–MS and their lower limits of quantification were much better (5 ng/mL), their method analyzed RIM in samples with large sample volumes (14 mL of blood), resulting in high costs. In addition, in mice at the p.o. dose of 10 mg/kg of [^{14}C]-RIM, the range of plasma concentration from 1 to 6 h varied between 0.3 and 0.015 μg/mL (Hoffman *et al.*, 1988). The measurable range of AMA and RIM by our method almost fully covers the previous values described above, indicating that our HPLC assay may be very suitable for the pharmakokinetic study of AMA using samples at a small volume (50 μL) and not using a radioactive compound.

Precision and accuracy

The intra-day and inter-day precision and accuracy for assay of AMA and RIM are shown in Table 1. In the intra-day assay, the range of standard deviations was 3.0–5.1%, and the recovery was 98.5–103.5% for AMA. For RIM, the range of standard deviation was 3.0–3.8%, and the recovery was 98.3–101.0%. In the inter-day assay, the range of the coefficient of variation was 3.9–7.6%, and the recovery was 98.5–105.3% for AMA. For RIM, the range of standard deviation was within 4.0–5.3%, and the recovery was 97.5–101.0%.

Application to pharmacokinetic study of AMA and RIM

The HPLC method was used to analyze plasma samples after a single i.v. or p.o. administration of AMA or RIM to rats. As shown in Fig. 4, concentration vs time profiles were constructed for up to 8 h for the analytes. Their pharmacokinetic parameters are listed in Table 2. The values of $MRT_{i.v.}$, CL_{tot} and Vd_{ss} of RIM were significantly higher than those of AMA. The values of $AUC_{i.v.,0\rightarrow 8\,h}$ and $AUC_{p.o.,0\rightarrow 8\,h}$ of RIM were significantly lower than those of AMA. The BA value of RIM was better than that of AMA. While

Table 1. Intra-day and inter-day assay precision and accuracy for the analysis of AMA and RIM from rat plasma

Concentration (μg/mL)		Measured (μg/mL) mean ± SD, $n = 4$	CV (%)	Recovery (%)
AMA				
0.04	Intra-assay	0.0414 ± 0.0021	5.1	103.5
0.2		0.197 ± 0.008	4.1	98.5
1		1.01 ± 0.03	3.0	101.0
0.04	Inter-assay	0.0421 ± 0.0032	7.6	105.3
0.2		0.197 ± 0.010	5.1	98.5
1		1.02 ± 0.04	3.9	102.0
RIM				
0.04	Intra-assay	0.0393 ± 0.0015	3.8	98.3
0.2		0.198 ± 0.006	3.0	99.0
1		1.01 ± 0.03	3.0	101.0
0.04	Inter-assay	0.0393 ± 0.0021	5.3	98.3
0.2		0.195 ± 0.008	4.1	97.5
1		1.01 ± 0.04	4.0	101.0

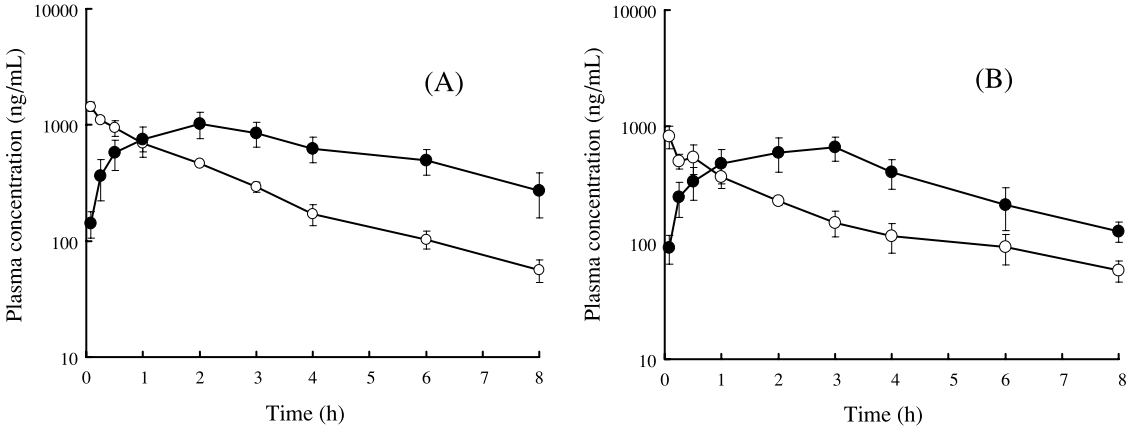

Figure 4. Plasma concentration-time courses of AMA and RIM after a single i.v. or p.o. administration of AMA or RIM in rats. (A) i.v. (6.0 mg/kg, $n = 5$) or p.o. (30 mg/kg, $n = 8$) administration of AMA; (B) i.v. (6.8 mg/kg, $n = 5$) or p.o. (34 mg/kg, $n = 8$) administration of RIM. Open and closed symbols showed i.v. and p.o. administrations, respectively. Each point represents the mean ± SD of five to eight rats.

Table 2. Pharmacokinetic parameters of AMA and RIM after a single i.v. or p.o. administration in rats

	AMA	RIM
i.v. administration (n = *5*)		
Dose (mg/kg)	6.0	6.8
$AUC_{0\to 8\,h}$ (μg × h/mL)	2.83 ± 0.19	1.59 ± 0.23*
$MRT_{i.v.}$ (h)	1.87 ± 0.10	2.15 ± 0.13*
CL_{tot} (L/h/kg)	2.13 ± 0.14	4.41 ± 0.68*
Vd_{ss} (L/kg)	3.98 ± 0.28	9.50 ± 1.66*
p.o. administration (n = *8*)		
Dose (mg/kg)	30	34
$AUC_{0\to 8\,h}$ (μg × h/mL)	4.93 ± 0.87	2.96 ± 0.42*
BA (%)	34.9	37.2
$MRT_{p.o.}$	3.49 ± 0.32	3.24 ± 0.28
C_{max} (μg/mL)	1.07 ± 0.11	0.755 ± 0.136*
T_{max} (h)	2.38 ± 0.52	2.63 ± 0.52

Each value represents the mean ± SD of five to eight rats. * $p < 0.05$ compared with AMA parameters.

the C_{max} value of RIM was significantly lower than that of AMA, a significant difference in the T_{max} or $MRT_{p.o.}$ values between AMA and RIM was not observed.

In rats, it was reported that the CL_{tot} and Vd_{ss} values of [^{3}H]-AMA were 1.2 L/h/kg and 6.4 L/kg, respectively (Goralski *et al.*, 1999). In humans, the plasma levels of AMA (100 mg p.o. dose) varied from 0.1 to 1.3 μg/mL, as reported by other authors (Biandrate *et al.*, 1972; Sioufi and Pommier, 1980; Belanger and Grech-Belanger, 1982). The recovery values of 92–95% of the dose of AMA were detected as the intact type in urine (Bleidner *et al.*, 1965), suggesting that BA of AMA should be more than 90%. To our knowledge, there was no information about the kinetic parameters of RIM in rats. In mice, the CL_{tot} and BA values of RIM were 4.3 L/h/kg and 58.6%, respectively, using [^{14}C]-RIM. In dogs, they were 3.7 L/h/kg and 99.4%, respectively (Hoffman *et al.*, 1988). In humans, the

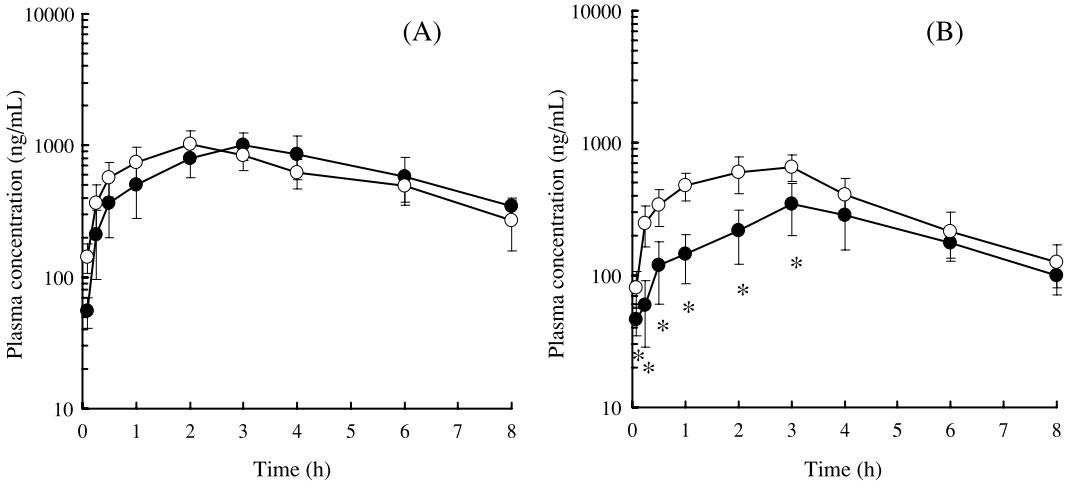

Figure 5. Plasma concentration-time courses of AMA and RIM after p.o. co-administration of AMA and RIM in rats. (A) Plasma concentration–time courses of AMA; (B) plasma concentration–time courses of RIM. (○) p.o. administration of AMA (30 mg/kg, $n = 8$) or RIM (34 mg/kg, $n = 8$) alone; (●) p.o. co-administration of AMA (30 mg/kg) and RIM (34 mg/kg, $n = 6$). Each point represents the mean ± SD of six to eight rats.

Table 3. Effects of RIM and AMA on pharmacokinetic parameters of AMA and RIM, respectively, in rats

	AMA		RIM	
	Without RIM ($n = 8$)	With RIM ($n = 6$)	Without AMA ($n = 8$)	With AMA ($n = 6$)
$AUC_{0\to8\,h}$ (μg × h/mL)	4.93 ± 0.87	5.17 ± 0.97	2.96 ± 0.42	1.60 ± 0.45*
$MRT_{p.o.}$	3.49 ± 0.32	3.51 ± 0.33	3.24 ± 0.28	3.71 ± 0.43
C_{max} (μg/mL)	1.07 ± 0.11	1.04 ± 0.24	0.755 ± 0.136	0.354 ± 0.155*
T_{max} (h)	2.38 ± 0.52	3.33 ± 0.82	2.63 ± 0.52	3.17 ± 0.41

Each value represents the mean ± SD of six to eight rats. * $p < 0.05$ compared with and without parameters.

plasma levels of RIM (100 mg p.o. dose) were from 0.005 to 0.8 μg/mL, with AUC values of approximately 3 μg × h/mL and T_{max} values within 2–6 h by previous data (Wills *et al.*, 1987; Holazo *et al.*, 1989). Thus, the species differences of AMA and RIM disposition kinetics were remarkably observed between the previous data and our data.

Pharmacokinetic drug–drug interaction between AMA and RIM

Figure 5A shows the mean plasma concentrations of AMA when given alone and in combination with AMA (30 mg/kg) and RIM (34 mg/kg) in the rats. The plasma AMA concentrations did not significantly change after simultaneous oral administration of AMA with RIM than after AMA alone. The pharmacokinetic parameters are listed in Table 3. No significant difference of expressed parameters was detected. Figure 5(B) shows the mean plasma concentrations of RIM when given alone and in combination with AMA (30 mg/kg) and RIM (34 mg/kg) in the rats. The plasma RIM concentrations were significantly lower at 0.083 at 3 h after simultaneous oral administration of RIM with AMA than after RIM alone. The values of $AUC_{0\to8\,h}$, and C_{max} of RIM significantly decreased to 54 and 47%, respectively, when AMA was co-administered. The change of the other parameters was not significant (Table 3). These data suggest that AMA may inhibit the intestinal transport of RIM *in vivo*, while RIM does not much affect that of AMA. Also, p.o. co-administration of AMA and RIM may result in the lack of pharmacological effects of RIM.

Inhibition of RIM for the transport of AMA was observed in transport kinetic studies of AMA and RIM through the blood–brain barrier in rats (Spector, 1988). Both IC_{50} values of AMA and RIM for the distribution of [^{14}C]-RIM and [^{3}H]-AMA, respectively, to the brain were within the range 1.0–2.5 mM. The previous data indicate that the affinity of AMA and RIM for the transport system(s) may be almost the same degree. However, our results show that AMA may inhibit intestinal absorption of RIM, suggesting AMA possesses the higher affinity for the transport system(s) involved

in its intestinal absorption than RIM. Also, it is possible that the different mechanisms from brain penetration are involved in the intestinal absorption of AMA and RIM.

Several rat organic cation transporters (rOCT1, rOCT1a, rOCT2 and rOCT3) have been molecularly identified and have been shown to be expressed in rat kidney (Grundemann *et al.*, 1994; Okuda *et al.*, 1996; Gorboulev *et al.*, 1997; Zhang *et al.*, 1997; Kekuda *et al.*, 1998). *In situ* hybridization and immunohistochemical evidence indicate that rOCT1 and rOCT2 are basolateral membrane transporters and are responsible for the first step in the secretion of organic cationic compounds in the proximal tubules. rOCT1, rOCT2 and other unknown transporters have been demonstrated to contribute to the transport of AMA for its renal secretion mechanism (Grundemann *et al.*, 1994; Urakami *et al.*, 1998; Budiman *et al.*, 2000; Karbach *et al.*, 2000). Thus, although the renal excretion mechanisms of AMA are becoming clearer at the molecular level, those of RIM are not investigated. rOCT1 and rOCT3 are expressed in the small intestine (Grundemann *et al.*, 1994; Kekuda *et al.*, 1998), and their localization in the small intestine is assumed to be in the basolateral membrane. In addition, these are likely to function primarily in the elimination of cationic drugs. However, the role of rOCT1 and rOCT3 in the intestinal transport of cationic drugs is poorly understood. Therefore, the data in this paper suggest that AMA may inhibit intestinal absorption of RIM via the unknown transporter(s) that function from the intestinal lumen into cells and/or exit enterocytes on the basolateral surface to reach the portal venous system and the systemic circulation. We expect our method to be very useful for solving the mechanisms for the intestinal absorption of AMA and RIM at molecular levels.

CONCLUSION

The present method is simple, rapid, sensitive and reproducible for simultaneously determining AMA and RIM concentrations in rat plasma. Also, this method is applicable to the pharmacokinetic studies of AMA and RIM in rats. Moreover, the p.o. co-administration of AMA and RIM to rats may result in the lack of pharmacological effects of RIM because AMA reduces the intestinal absorption of RIM. We strongly expect our method to be suited not only to therapeutic drug monitoring, but also to resolving the intestinal absorption mechanisms of AMA and RIM.

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