In Vitro Plasma Metabolism of RMP-7

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Received March 4, 1994; accepted May 20, 1994
KEY WORDS: RMP-7; bradykinin; plasma metabolism, angiotensin converting enzyme.

INTRODUCTION

RMP-7, a bradykinin B_2 receptor-mediated blood brain barrier permeabilizer, enhanced the delivery of molecules to tumors implanted into the brain (1). RMP-7 is being evaluated for its ability to enhance delivery of drugs to the central nervous system for the treatment of diseases such as cancer and opportunistic infections associated with AIDS.

The metabolism of bradykinin has been described (2–8). RMP-7 (H-Arg-Pro-Hyp-Thi-Gly-Ser-Pro-4-MeTyr-$\Psi(CH_2NH)$-Arg-OH; Thi = thienylalanine) was designed to minimize degradation by angiotensin converting enzyme (the primary enzyme responsible for degradation of bradykinin in the lung) and carboxypeptidases (the main enzymes responsible for the degradation of bradykinin in plasma). Prior to studies of *in vivo* metabolism, the *in vitro* metabolism of RMP-7 was examined.

MATERIALS AND METHODS

Materials

Plasma from Sprague-Dawley rats, Hartley guinea pigs, New Zealand Albino rabbits and beagle dogs was used within one day of collection. RMP-7 was prepared for Alkermes by Peninsula Laboratories, Inc., Belmont, California. Bradykinin acetate was purchased from Bachem Bioscience, Inc., Philadelphia, Pennsylvania. Angiotensin converting enzyme was obtained from Sigma Chemical Company, St. Louis Missouri.

HPLC Analysis

HPLC System A: Hewlett-Packard 1090M; Vydac C18 Protein & Peptide column (4.6 mm × 250 mm); Eluant A (0.1 M $NaClO_4$/0.1% H_3PO_4 (85%); pH 2.5); Eluant B (acetonitrile); Gradient (10% B to 40% B over 30 minutes); 1.2 mL/min; 210 nm monitored.

HPLC System B: Applied Biosystems 1000S; Bondclone 10 C18 column (3.9 mm × 300 mm); Eluant A: 0.1% TFA, Eluant B: acetonitrile. Gradient: 14% B for 10 minutes, step to 23% B over 1 min, and then 23% B for 19 minutes; 1 mL/min.

HPLC System C: Applied Biosystems 1000S; Bondclone 10 C18 column (3.9 mm × 3000 mm); Eluant: 17% acetonitrile/0.1% TFA; 1 mL/min.

HPLC System D: Beckman gradient HPLC; Waters μBondapak C18 column (3.9 mm × 300 mm), Phenomenex Bondclone 10 C18 guard (3.9 × 30 mm); Eluant A (0.1% TFA); Eluant B (0.65% TFA in acetonitrile); Gradient (20% B to 50% B over 15 minutes); 1 mL/min, 210 nm monitored.

Metabolism of RMP-7

Typical protocols are described below.

20% Plasma

Plasma from four animals was used, except for male rabbits (n = 3), female humans (n = 1), and male humans (n = 3). Studies were initiated at room temperature, except for guinea pig studies, in which solutions were pre-warmed to 37°C.

PBS (540 μL), RMP-7 (61 μL; 2 mg/mL in PBS) and rabbit plasma (150 μL) were incubated at 37°C. Aliquots (100 μL) were withdrawn periodically. 10% HCl (22 μL) and water (97 μL) were added, and the samples centrifuged. Bradykinin (25 μL; 0.2 mg/mL in water), as internal standard, was added to the supernatant. The samples were analyzed (HPLC System A).

88% Plasma

Samples of RMP-7 (49 μL in PBS at the following concentrations: 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL and 0.61 mg/mL) were mixed with rabbit plasma (351 μL), and incubated at 37°C. Aliquots (50 μL) were withdrawn periodically. HCl (10%; 11 μL), an internal standard (bradykinin, 25 μL; 0.2 mg/mL in water) and additional water (97 μL) were added. The samples were centrifuged. The supernatants were analyzed (HPLC System A).

Data Analysis

Concentrations of RMP-7 were determined using peak areas relative to a standard curve, and were normalized using the internal standard (bradykinin). Values of $t_{1/2}$ were derived from first-order kinetics. Statistical analysis of differences was performed using an unpaired two-tailed t-test. V_{max} and K_m values were derived from Michaelis-Menten kinetics.

Isolation of Metabolites from Rat Plasma

First Metabolite: RMP-7 (1.7 mg/mL in saline; 576 μL), 3XPBS (264 μL) and rat plasma (60 μL) were incubated at 37°C for 4 hours. 10% HCl (100 μL) was added. The metabolite was isolated using HPLC Systems B and C, and analyzed by FAB-MS.

The second metabolite was generated and isolated similarly to the first metabolite, except the following solutions were used: RMP-7 (655 μg in 140 μL water), 1X PBS (100 μL) and rat plasma (60 μL). FAB-MS and amino acid analysis were performed.

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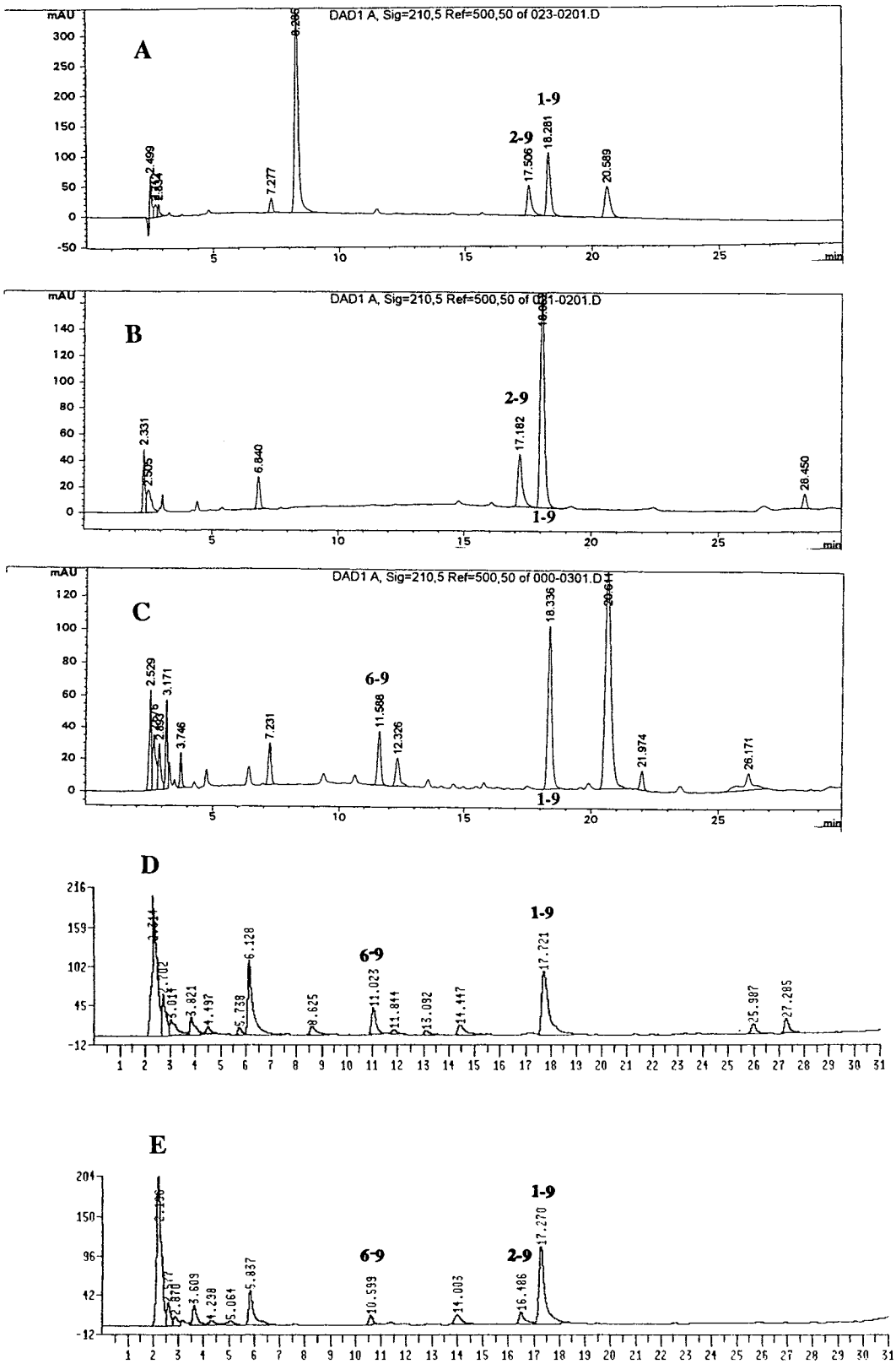

Fig. 1. HPLC chromatograms showing the positions of the metabolites of RMP-7 in 20% plasma. (A) Rat plasma. (B) Guinea pig plasma. (C) Rabbit plasma. (D) Dog plasma. (E) Human plasma. Compound 1–9 is RMP-7. Compound 2–9 is *des*-Arg^1-RMP-7. Compound 6–9 is the C-terminal tetrapeptide derived from RMP-7. In A and C, a peak associated with plasma was the largest peak observed in the chromatograms.

Angiotensin Converting Enzyme

RMP-7 (0.2 mg/mL in PBS, 5 μL), PBS (14.2 μL), and angiotensin converting enzyme (0.8 μL; 0.0008 U enzyme activity) were incubated at room temperature (analyzed using HPLC System D). Bradykinin was assayed under comparable conditions.

RESULTS AND DISCUSSION

The *in vitro* metabolism of RMP-7 in plasma was studied. Inter-species, intra-species and gender differences were examined using 20% plasma (Table 1). The relative order of rates observed was guinea pigs > rats > humans > dogs = rabbits. The standard deviations were large for all species except the guinea pig. The rat was the only species for which the study was initiated on multiple days. The standard deviations were small on a single day, but larger when data from all days were combined. It is possible that this was the result of the rats purchased on a given day being more closely genetically related than rats purchased over multiple days. The only species in which a statistically significant gender difference was observed was the rat.

Detailed kinetic analysis was performed using 88% plasma. Concentrations ranged from 0.07 mM to 1 mM (HPLC quantitation range). Double-reciprocal plots were used to determine apparent K_m and V_{max} values (Table 2). RMP-7 loss appeared to obey Michealis-Menten kinetics, indicating that the observed RMP-7 breakdown was mediated by enzymatic processes. Differences between species in V_{max} were larger than differences in K_m.

Metabolites formed in rat plasma were isolated and identified. The first observed metabolite was determined to be *des*-Arg^1-RMP-7 (FAB-MS MH^+ = 942). The second observed metabolite was determined to be the C-terminal tetrapeptide fragment (H-Ser-Pro-4-Me-TyrΨ(CH_2NH)Arg-OH) using amino acid analysis (Ser and Pro observed) and FAB-MS (MH^+ = 522).

Metabolism patterns in other species were compared (Figure 1). In guinea pig plasma, *des*-Arg^1-RMP-7 built-up and then was metabolized to the C-terminal tetrapeptide (as with rat plasma). In human plasma, formation of *des*-Arg^1-RMP-7 and the C-terminal tetrapeptide occurred at similar rates. In dog and rabbit plasma, only the C-terminal tetrapeptide metabolite was observed. Based on the relative rates

Table I. Half-life of RMP-7 in 20% Plasma

Species	Gender	$t_{1/2}$ (min)
Rat (day 1)	female	15.6 ± 3.9
Rat (day 2)	female	17.9 ± 1.9
Rat (day 3)	female	32.9 ± 2.4
Rat (day 2)	male	65.1 ± 30.3
Rat (day 3)	male	89.7 ± 20.4
Guinea Pig	female	2.2 ± 0.1
Guinea Pig	male	2.3 ± 0.3
Rabbit	female	2402 ± 632
Rabbit	male	1337 ± 721
Dog	female	1856 ± 251
Dog	male	1352 ± 700
Human	female	361
Human	male	640 ± 313

Table II. K_m and V_{max} for the *in Vitro* Metabolism of RMP-7 in 88% Plasma

Species	K_m (μM)	V_{max} (μM min^{-1})
rat (female 1)	454	26.25
rat (female 2)[1]	403	11.46
rat (male)	207	3.64
guinea pig (female)	302	107.98
guinea pig (male)	782	324.87
rabbit (female)	361	0.46
rabbit (male)	282	0.27
human (female)	87	0.81

[1] Plasma from two individual rat females was studied to determine K_m and V_{max}. The data for other species were obtained using plasma from one animal.

and the pattern of metabolites, a significant portion of the inter-species differences might be attributed to different amounts (or activities) of the enzyme responsible for the N-terminal arginine cleavage. It is possible that, in all species, the first cleavage is the N-terminal arginine, which is required for further enzymatic processing.

We also studied the cleavage of RMP-7 by angiotensin converting enzyme (ACE). RMP-7 was less susceptible to ACE than was bradykinin. Under the conditions used, bradykinin was completely metabolized by ACE in 74 min., whereas 68% of RMP-7 was intact after 18 hours. Other studies of RMP-7 cleavage by enzymes (9) revealed that thienylalanine was liberated from RMP-7 upon extended exposure to ACE. This was not expected, and indicated that the metabolism of RMP-7 by ACE was due (at least in part) to an impurity in the enzyme preparation.

RMP-7 was designed to limit metabolism by carboxypeptidases and ACE. Carboxypeptidases, a key component of *in vitro* plasma metabolism of bradykinin, cannot cleave the reduced peptide bond between 4-Me-Tyr^8 and Arg^9. Therefore, the *in vitro* plasma metabolism profile of RMP-7 is considerably different from that reported for bradykinin. The reduced peptide bond of RMP-7 also appears to interfere with the ability of ACE to cleave RMP-7. The amino-terminus of RMP-7 was not designed to resist metabolism; therefore the formation of *des*-Arg^1-RMP-7 was observed. Other analogs have been designed for improved stability at the amino-terminus (*e.g.* D-Arg^0-RMP-7); however, their effects *in vitro* and *in vivo* have not yet been addressed.

ACKNOWLEDGMENTS

The authors thank William Kelley, Gary Musso, Carol Gloff, Janet Smart and Pravin Chaturvedi for their helpful discussions.

REFERENCES

1. T. Inamura, T. Nomura, R. T. Bartus, and K. L. Black. Intracarotid infusions of RMP7, a bradykinin analog: a method for selective drug delivery to brain tumors. *J. Neurosurgery*, in press.
2. I. A. Sheikh and A. P. Kaplan. Studies of the digestion of bradykinin, lys-bradykinin, and *des*-Arg^9-bradykinin by angiotensin converting enzyme. *Biochem. Pharmac.*, 35, 1951–1956 (1986).
3. I. A. Sheikh and A. P. Kaplan. Studies of the digestion of bradykinin, lysyl bradykinin, and kinin-degradation products by car-

boxypeptidases A, B, and N. *Biochem. Pharmac.*, **35**, 1957–1963 (1986).
4. Y. S. Bakhle. Pulmonary metabolism of bradykinin analogues and the contribution of angiotensin converting enzyme to bradykinin inactivation in isolated lungs. *Br. J. Pharmacol.*, **59**, 123–128 (1977).
5. F. Marceau, M. Gendreau, J. Barabe, S. St.-Pierra, and D.-Regoli. The degradation of bradykinin (BK) and of *des*-Arg^9-BK in plasma. *Can. J. Physiol. Pharmacol.*, **59**, 131–138 (1981).
6. D. A. McCarthy, D. E. Potter and E. D. Nicolaides. An *in vivo* estimation of the potencies and half-lives of synthetic bradykinin and kallidin. *J. Pharmacol. Exp. Ther.*, **148**, 117–122, 1965.
7. G. Drapeau, N. E. Rhaleb, S. Dion, D. Jukic and D. Regoli. [Phe^8(CH_2NH)Arg^9]bradykinin, a B_2 receptor selective agonist which is not broken down by either kininase I or II. *Eur. J. Pharmacol.*, **155**, 193–195 (1988).
8. G. Drapeau, A. Chow, and P. E. Ward. Metabolism of bradykinin by angiotensin I converting enzyme and carboxypeptidase N. *Peptides*, **12**, 631–638 (1991).
9. J. A. Straub, A. Akiyama, G. Musso, P. Parmar. Limited enzymatic digestion for the determination of the ratio of quantities of minor diastereomeric contaminants in preparations of RMP-7, a peptide containing a reduced peptide bond. (manuscript in preparation)