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Limited enzymatic digestion for the determination of the quantities of minor diastereomeric impurities in preparations of RMP-7, a peptide containing a reduced peptide bond

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Abstract

RMP-7 is a bradykinin analogue containing all "L" amino acids and a reduced dipeptide bond between amino acids eight and nine. This reduced dipeptide bond [4-Me-Tyr-Ψ(CH_2NH)-Arg] is created under synthetic conditions which could result in inversions of the chiral centers of either 4-Me-Tyr or Arg. Stereoisomers of RMP-7 would be expected to have altered biological specificity. Current chromatographic methods are not sufficiently sensitive to distinguish the anticipated stereoisomeric variants of the intact molecule. Therefore we have devised an analytical method based on limited enzymatic digestion of the compound followed by reversed-phase HPLC analysis of the peptide fragments. Using this method we have been able to carry out precise and reliable quantitative analysis of the stereoisomeric content of different batches of peptide prepared for biological testing.

1. Introduction

RMP-7 [H-Arg-Pro-Hyp-Gly-Thi-Ser-Pro-4-Me-Tyr-Ψ(CH_2NH)-Arg-OH; Thi = thienylalanine] is an analogue of the vasodilatory hormone bradykinin. Biological activity of bradykinin is known to be triggered by interaction with a specific cell surface receptor molecule. The kinetics of elimination of bradykinin are influenced by enzymatic digestion of the molecule in circulation. The RMP-7 molecule is a synthetic agonist of the naturally occurring compound designed to interact with the normal bradykinin B_2 receptor and to be more resistant to enzymatic degradation [1]. We are currently evaluating RMP-7 as a receptor mediated permeabilizer of the blood–brain barrier. Our strategy is to administer RMP-7 in combination with therapeutic molecules to which the blood–brain barrier would otherwise be impermeable. Working as an adjuvant, RMP-7 is intended to assist in delivery of these molecules to the brain or brain tumors in order to treat specific disease states [2].

A key structural feature of RMP-7 is the reduced peptide bond, 4-Me-Tyr-Ψ(CH_2NH)-Arg which was incorporated into the sequence in order to increase metabolic stability and receptor specificity. This strategy has been used for other biologically active peptides such as somatostatin [3] and tetragastrin [4]. Key to the overall strategy is the targeting of such molecules to specific biological receptors. Implicit in any such model is the assumption that only one stereo-

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isomer will have the specificity to result in biological potency. The presence of varying amounts of other stereoisomers in individual batches of material prepared for biological testing could result in quantitative variations in response or therapeutic effect.

We have devised a synthesis of the overall molecule in which reductive amination of a Boc-protected amino aldehyde with cyanoborohydride forms the reduced peptide bond isostere [5,6]. Reaction conditions at this step of the synthesis are sufficiently vigorous that epimerization of the key chiral centers might be expected for several reasons, including (1) reported lability of amino aldehydes [7], (2) sensitivity of the imine formed in situ during the reductive amination [8] and (3) inversion of the C-terminal arginine upon esterification to the resin or following HF cleavage

Since we expect the pharmacologically active stereoisomer of RMP-7 to be that with all L-amino acid constituents, epimerization of any single chiral center might lead to undesirable isomers. Centers 8 and 9 are those of most concern and we have conducted several studies of reaction strategies designed to minimize inversions at these carbons. In order to measure the effectiveness of our synthetic strategies we needed a sensitive quantitative analytical method to detect the relative amounts of the three major stereoisomers: 8L,9L; 8D,9L; and 8L,9D. To assist in our analysis we synthesized the two major putative impurities which we labeled RMP-10 and RMP-12:

RMP-7: all L chiral centers;

RMP-10: 8D,9L;

RMP-12: 8L,9D.

We anticipated that few, if any molecules would be formed by inversion of both chiral centers and we have never found evidence for the 8D,9D stereoisomer.

Analysis of batches of RMP-7 prepared during scale-up for clinical trial testing required validation of the quantitative method for detecting the levels of the two contaminating stereoisomers in the presence of the major component. We did not find an analytical HPLC method capable of resolving mixtures of all three stereoisomers. Acid or enzymatic digestion of the peptides followed by chromatography on chiral supports could be used to assay the purity of the individual amino acids [9–11]. Such a method might have been used to generate the reduced dipeptide and measure the ratios of the three anticipated species. However, acid hydrolysis can cause racemization itself and reliable measurements based on this method must be assessed by GC–MS technology which was not readily available. We were also concerned that the reduced dipeptide fragment formed by such digestion might not be suitable for derivatization for GC analysis. Therefore, we devised a method for enzymatic digestion coupled to HPLC analysis.

We considered two enzyme/HPLC-based strategies: (1) complete enzymatic digestion of RMP-7 to amino acids followed by chiral chromatography and (2) partial digestion to peptide fragments followed by non-chiral chromatography. We examined enzymes known to degrade the parent molecule (bradykinin; H–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg–OH), and enzymes which act as general peptidases. These included angiotensin-converting enzyme (ACE; cleaves bonds 7–8 and 5–6 of bradykinin), prolidase (cleaves bond 1–2 of bradykinin) and α-chymotrypsin (cleaves bonds 8–9 and 5–6 of bradykinin) [12], as well as aminopeptidase M [13], papain [14], pepsin [15], subtilisin [16] and pronase [9]. In this report, we detail our findings with respect to the applicability of these enzymes for use in limited proteolysis of RMP-7. In addition, we report our analysis of various batches of RMP-7 when analyzed by limited proteolysis with pronase followed by HPLC analysis.

2. Experimental

2.1. Materials

RMP-7 triacetate salt was prepared for Alkermes by Peninsula Labs. (Belmont CA, USA) using solid-phase peptide synthesis on a Merrifield resin using the N^{α}-Boc protecting group strategy. The reduced peptide bond between

residues 8 and 9 was initially synthesized according to the method described by Coy and Sasaki (synthetic route 1) [5]. In later lots of RMP-7, the reduced peptide bond was synthesized according to the method of Ho et al. (synthetic route 2) [6]. Authentic samples of 8D,9L-RMP-7 (RMP-10 trifluoroacetate salt, lyophilized powder) and 8L,9D-RMP-7 (RMP-12 trifluoroacetate salt, lyophilized powder) were prepared for Alkermes by Peninsula Labs. using solid-phase peptide synthesis. ACE (rabbit lung), α-chymotrypsin (bovine pancreas), prolidase (porcine kidney), papain (*Papaya latex*), pepsin (porcine stomach mucosa), and subtilisin Carlsberg (*Bacillus licheniformis*) were purchased from Sigma (St. Louis, MO, USA). Aminopeptidase M (hog kidney) was purchased from Pierce (Rockford IL, USA). Pronase (Protease, non-specific from *Streptomyces griseus*) was purchased from Boehringer Mannheim Biochemicals. Phosphate-buffered saline (PBS) consisted of 1.15 g Na_2HPO_4 (8.1 m*M*), 0.26 g $NaH_2PO_4 \cdot H_2O$ (1.9 m*M*), 0.20 g KCl (2.7 m*M*) and 7.0 g NaCl (120 m*M*) in 1 l water (Milli-Q).

2.2. Enzymatic methods

Angiotensin-converting enzyme

RMP-7 (4 μg/μl, 25 μl), PBS (375 μl) and ACE (0.1 U in 100 μl PBS) were mixed and incubated at 37°C for 21.5 h. The reaction mixture was assayed using HPLC system B.

α-Chymotrypsin

RMP-7 (1 μg/μl solution in saline, 25 μl), buffer (0.2 *M* triethylammonium acetate, pH 8.0, 100 μl) and α-chymotrypsin (1.1 U enzyme activity in 25 μl buffer) were mixed and incubated at 37°C for a total of 25 h. The reaction mixture was assayed using HPLC system A.

Prolidase

A solution of manganese chloride (1.0 *M*, 400 μl), glutathione (reduced form, 0.03 *M*, 100 μl) and buffer [0.1 *M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 8.0, 2.4 ml] was made. RMP-7 (2.0 μg/μl solution in saline, 10 μl), buffer (0.1 *M* HEPES, pH 8.0, 165 μl) and prolidase (25 μl, 53.4 U enzyme activity) were mixed. A 100-μl aliquot of the RMP-7/prolidase solution was mixed with 100 μl of manganese chloride/glutathione solution. The resulting mixture allowed to stand at room temperature for 30 min, and was then assayed using HPLC system C.

Papain

RMP-7 (4 μg/μl solution in water, 25 μl), buffer (0.1 *M* ammonium acetate, pH 5.7, 454 μl), mercaptoethanol (diluted 1:32 with water, 5 μl) and papain (21 μl; 10 U enzyme activity) were mixed and incubated at 37°C at 22.5 h. The reaction mixture was assayed using HPLC system B.

Pepsin

RMP-7 (4 μg/μl solution in water, 25 μl), buffer (24 m*M* citrate, pH 4.6, 471 μl) and pepsin (11.8 U enzyme activity in 4 μl buffer) were mixed and incubated at 37°C for 24 h. The reaction mixture was assayed using HPLC system B.

Subtilisin

RMP-7 (4 μg/μl, 25 ml), PBS (345 μl) and subtilisin Carlsberg (1.0 U enzyme activity in 130 μl buffer) were mixed and incubated at 37°C for 23.5 h. The reaction mixture was assayed using HPLC system B.

Aminopeptidase M

RMP-7 (1.0 μg/μl solution in saline, 25 μl), buffer (0.1 *M* HEPES, pH 8.5, 25 μl), glutathione (reduced form, 9.2 mg/ml solution in saline, 25 μl) and aminopeptidase M solution (50 μl, 1 U enzyme activity) were mixed and incubated at 37°C for a total of 17 days. The reaction mixture was assayed periodically using HPLC system A.

Pronase

RMP-7 drug substance (1 μg/μl in water, 100 μl), Tris–calcium chloride dihydrate buffer (53.3 μl; 0.2 *M* Tris, 5 m*M* calcium chloride, pH adjusted to 7.5 with 1 *M* HCl) and pronase (9.3 μl of a solution of 5.2 mg solid dissolved in 10 ml

Tris–calcium chloride dihydrate buffer; 0.56 U enzyme activity) were mixed and incubated at 37°C for 24 h. HPLC system A was used for initial studies of cleavage patterns. HPLC systems D and E were used for analysis of diastereomeric ratio. RMP-12 was analyzed using the same conditions as were used for RMP-7. Due to limited sample availability, when RMP-10 was analyzed, a concentration of 0.25 μg/μl in water was used in place of the 1 μg/μl solution used for RMP-7.

2.3. HPLC Analysis

System A: A Hewlett-Packard 1090 HPLC system with a diode-array detector set at 210, 214, 230 and 280 nm; a Vydac C_{18} protein and peptide column (5 μm particle size, 300 Å average pore diameter, 250 mm × 4.6 mm); a linear gradient system of 10% eluent B to 40% eluent B over 30 min (eluent A: 0.1 *M* aqueous sodium perchlorate/0.1% phosphoric acid (85%), pH 2.5; eluent B: acetonitrile); flow-rate: 1 ml/min.

System B: HP 1090 system with a diode-array detector set at 210, 214, 230 and 280 nm; a Phenomenex Bondclone C_{18} column (300 mm × 3.9 mm); a linear gradient system of 0% eluent B to 39% eluent B over 39 min (eluent A: 0.1 *M* aqueous sodium perchlorate/0.1% phosphoric acid (85%), pH 2.5; eluent B: acetonitrile); flow-rate: 1 ml/min.

System C: a Beckman system with a Model 166 UV detector set at 210 nm; a Waters μBondapak C_{18} column (300 mm × 3.9 mm, 10 μm particle size); a linear gradient system of 10% eluent B to 40% eluent B over 30 min [eluent A: 0.1% trifluoroacetic acid in water; eluent B: acetonitrile]; flow-rate: 1 ml/min.

System D: HP 1090 system with a diode-array detector set at 210 nm; a Vydac C_{18} protein and peptide column (5 μm particle size, 300 Å average pore diameter, 250 mm × 4.6 mm); an isocratic elution system [96% eluent A and 4% eluent B; eluent A: 0.2% aqueous triethylamine, pH adjusted to 5.2 with phosphoric acid (85%); eluent B: MeOH]; flow-rate: 1 ml/min; 45 min run time.

System E: HP 1090 Series II system with a diode-array detector set at 210 nm; a Zorbax 300SB-C_8 (5 μm particle size, 300 Å average pore diameter, 250 mm × 4.6 mm); an isocratic elution system (0.2% aqueous triethylamine, pH adjusted to 3.1 with phosphoric acid); flow-rate: 1 ml/min; 45 min run time.

3. Results and discussion

Initially we evaluated the fragmentation pattern resulting from incubation of RMP-7 with various enzymes. Digestion conditions were based on procedures recommended by the supplier of each enzyme. The reaction progress was monitored over time by HPLC. For practical purposes, 1–3 days incubation times were used. Aminopeptidase M was monitored over 17 days, because the digestion appeared to be incomplete at earlier times.

These studies used HPLC analysis on C_{18} reversed-phase columns with UV detection to determine the extent of digestion of RMP-7 by various enzymes. The structures of the fragments of RMP-7 produced by enzymatic digestion were assigned based on UV spectral data, and a comparison of HPLC retention times of these fragments to the retention times of known structures derived from RMP-7. Monitoring the enzymatic digestions using a diode-array detector at multiple wavelengths that included 280 nm allowed us to identify HPLC peaks associated with fragments containing 4-Me–Tyr–$\Psi(CH_2NH)$–Arg (in RMP-7, 4-Me–Tyr λ_{max} = 274 nm). The retention times of des-Arg^1–RMP-7 and the C-terminal tetrapeptide [H–Ser–Pro–4-Me–Tyr–$\Psi(CH_2NH)$–Arg–OH] were known, as these compounds had been isolated by HPLC and identified by fast atom bombardment MS analysis, as a part of a study of the metabolism of RMP-7 [1]. Authentic C-terminal dipeptide [H–4-Me–Tyr–$\Psi(CH_2NH)$–Arg–OH] had been synthesized previously. Thienylalanine was the only free amino acid that could be easily detected in the HPLC systems used, as it has a characteristic 230 nm absorbance, and is sufficiently hydrophobic to be retained on the col-

umn. Any fragment of RMP-7 with a significant absorbance at 230 nm, but not at 280 nm, was assumed to contain thienylalanine but not 4-Me–Tyr.

Observed enzymatic cleavage sites of RMP-7 are summarized in Fig. 1. Only aminopeptidase M appeared able to hydrolyze RMP-7 to constituent amino acids and the C-terminal reduced dipeptide [H-4-Me–Tyr^8–$\Psi(CH_2NH)$–Arg–OH; $t_R = 5.5$ min in system A). However, this digestion was incomplete after 17 days, and the observed remaining C-terminal tetrapeptide resulted from one (or both) of the minor stereoisomeric impurities (RMP-10 and/or RMP-12) in the preparation of RMP-7 (the C-terminal tetrapeptide resulting from RMP-7 had been completely digested). Prolidase cleaved the Arg^1–Pro^2 bond resulting in the formation of des-Arg^1–RMP-7 ($t_R = 21.0$ min in system C). Enzymatic digestions employing ACE, α-chymotrypsin, papain or pronase resulted in the formation of diastereomeric C-terminal tetrapeptides, H-Ser–Pro–4-Me–Tyr–$\Psi(CH_2NH)$–Arg–OH [$t_R = 11.6$ min (8L,9L isomer) and 12.5 min (8D,9L and 8L,9D isomers) in system A; $t_R = 23.0$ min (8L,9L isomer) and 24.0 min (8D,9L and 8L,9D isomers) in system B]. With ACE, papain or pronase, the remainder of RMP-7 appeared to be digested to single amino acids, since free thienylalanine was detected in the analyte. Based on substrate specificity, free thienylalanine was not expected to be formed in the digestions using

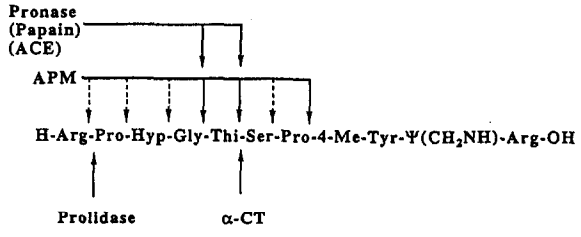

Fig. 1. Sites of enzymatic cleavage of RMP-7. APM = Aminopeptidase M; α-CT = α-chymotrypsin; ACE = angiotensin-converting enzyme. Solid lines indicate sites of cleavage detected directly via product analysis. Dashed lines indicate expected sites of cleavage that were not directly observed via product analysis. ACE and papain are in parentheses as the observed sites of cleavage were not predicted based on the site specificity of these enzymes.

ACE or papain. It is possible that the observed hydrolysis of RMP-7 with ACE or papain was the result of impurities in the enzyme preparations. Only α-chymotrypsin appeared to have a single cleavage site at the Thi^5–Ser^6 bond, which resulted in the formation of the diastereomeric C-terminal tetrapeptides along with the N-terminal pentapeptide ($t_R = 8.5$ min in system A). Pepsin and subtilisin did not cleave RMP-7.

Since the C-terminal tetrapeptides were small fragments in which the single-center epimers of interest are diastereomers rather than enantiomers, we concentrated on their separation. Based on the initial studies, the pronase reaction appeared to be a simple and clean method of generating the tetrapeptides; therefore, all later separation studies were performed with pronase digests. In addition to RMP-7, these studies used samples of authentic RMP-10 and RMP-12 as standards. These molecules appeared to be completely digested to the C-terminal tetrapeptides by pronase under the conditions used.

Studies of non-chiral HPLC conditions for separation of the tetrapeptides were performed. The first system found to separate all three isomeric C-terminal tetrapeptides derived from RMP-7 used a Vydac C_{18} column (system D; 8L,9L derived peak $t_R = 26$ min; 8L,9D derived peak $t_R = 31$ min; 8D,9L derived peak $t_R = 34$ min). Unfortunately, quantification of small percentages of the 8L,9D isomer was problematic, since the peaks were broad and the peak from the 8L,9L isomer frequently tailed into the 8L,9D derived peak. The tetrapeptides were composed of one primary amine, one secondary amine, one guanidinium group, and one carboxylic acid moiety, resulting in an overall charge of +2. Such basic molecules tend to tail on reversed-phase chromatographic systems due to ionic interactions with incompletely capped silica. Therefore, other columns reported by their suppliers to be good for separation of basic molecules (such as the Zorbax 300SB-C_8 used in HPLC system E) were examined. HPLC system E resulted in improved separation of all three diastereomeric C-terminal tetrapeptides, as illustrated in Fig. 2.

This method has been subsequently used to

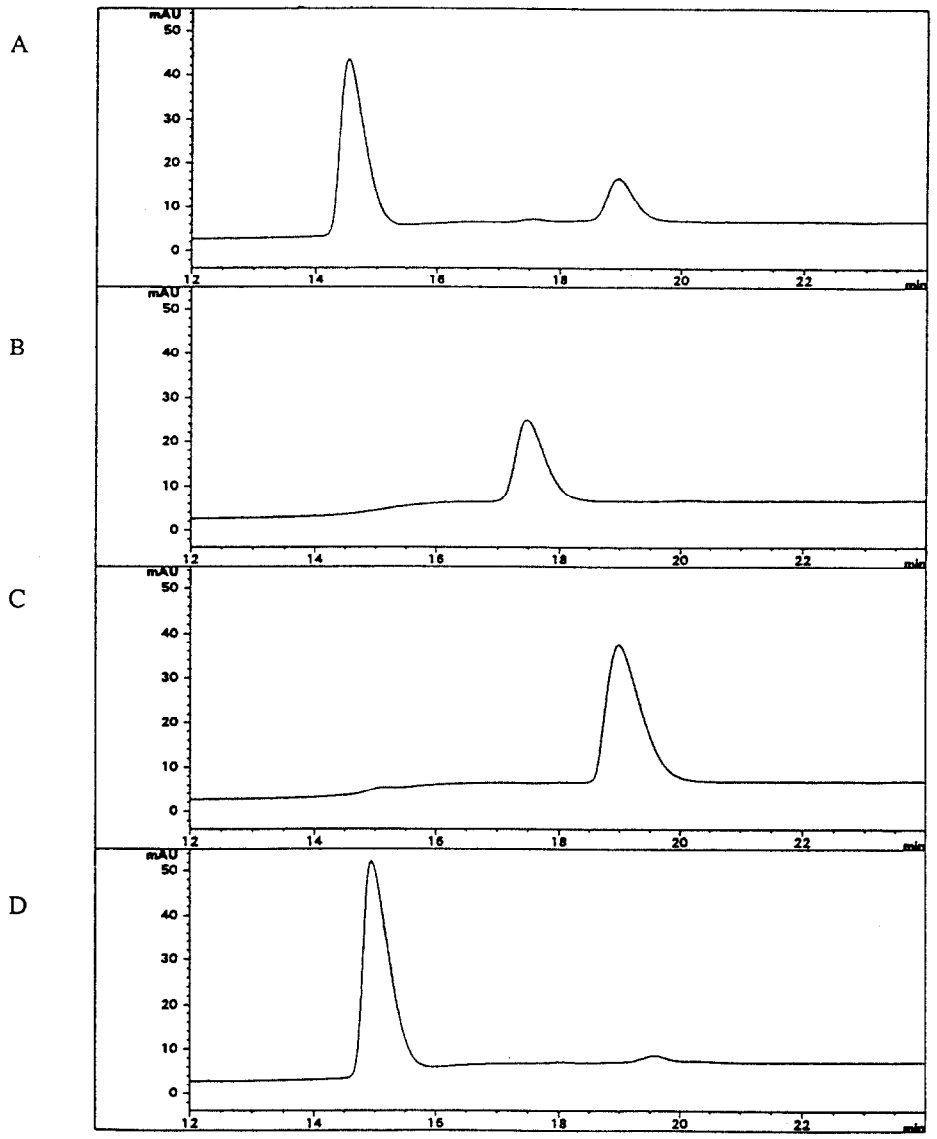

Fig. 2. HPLC analysis using system E. (A) RMP-7 (via synthetic route 1) treated with pronase. (B) RMP-10 treated with pronase. (C) RMP-12 treated with pronase. (D) RMP-7 (via synthetic route 2) treated with pronase. *y*-Axes: absorbance at 210 nm.

assess the ratios of diastereomers present in various batches of RMP-7. The 8D,9L-diastereomer was the major undesired isomer in the early lots (produced using synthetic route 1) as shown in Fig. 2A. This demonstrated that the reductive amination used to prepare the reduced peptide bond between residue 8 and 9 resulted in significant epimerization at the 8 position. This knowledge led to modifications of the synthetic process to minimize the presence of the undesired diastereomers [6]. Later lots (produced using synthetic route 2) had significantly reduced amounts of the 8D,9L isomer, and only trace amounts of the 8L,9D isomer (Fig. 2D).

This paper demonstrates the use of limited enzymatic degradation of a peptide (RMP-7) to determine the ratios of diastereomers present in the peptide. Such an analysis is important for

those peptides whose manufacturing process might result in the presence of diastereomers. While the strategy of limited enzymatic digestion is not a general method of analysis, the limited digestions can be particularly useful in cases of peptides containing unusual components such as the reduced dipeptide bond in RMP-7. The limited digestions also have the advantage in that they employ non-chiral column supports and equipment that is present in most analytical laboratories.

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