Chemical Pathways of Degradation of the Bradykinin Analog, RMP-7

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INTRODUCTION

RMP-7 (Arg-Pro-Hyp-Gly-Thi-Ser-Pro-4-MeTyrΨ(CH_2NH)Arg) is a bradykinin B_2 receptor agonist, which is being evaluated for its ability to facilitate entry of therapeutics into the brain for treatment of a number of diseases (1). It is currently in clinical trials for the treatment of such diseases as cryptococcal meningitis (in combination with amphotericin B) and brain tumor (in combination with carboplatin).

Our objective was to develop a formulation for clinical trials. We anticipated the stability would resemble that of other peptides (2–6). The degradation pathways of RMP-7 and pH/buffer stability profiles were studied. A clinical formulation was then chosen and evaluated.

MATERIALS AND METHODS

Materials

RMP-7 triacetate salt was prepared for Alkermes by Peninsula Laboratories, Inc. (Belmont, California). N-Ac^0-RMP-7 trifluoroacetate salt was prepared at Alkermes.

Thermal Degradants of RMP-7 Solid

Samples of RMP-7 drug substance (solid) were stored at 70°C for 1 day for degradant isolation purposes, and at 40°C for 115 days for use as analytical standards.

Degradants were isolated by preparative HPLC (HPLC Methods C, D or E). Any degradant isolated using Method C was subjected to buffer exchange (0.1% TFA in water containing 55% acetonitrile on a C-18 Sep-Pak) prior to lyophilization. The isolated degradants were analyzed as follows: 1) FAB-MS and electrospray MS (samples were analyzed at M-Scan, West Chester, PA), 2) HPLC on Systems B and C with UV spectrum of peak taken from the HPLC, 3) amino acid analysis (samples were analyzed using a Waters Pico Tag amino acid analyzer at Analytical Biotechnology Services, Boston, MA), and 4) co-elution on HPLC systems A and B with an authentic sample (used for N-Ac^0-RMP-7).

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Kinetic Studies

Samples were prepared as described below. At each time-point samples were analyzed by HPLC immediately. Time-points employed for the studies at 72°C were 0, 1 week, 2 weeks, 3 weeks, 4 weeks, and 6 weeks. Time-points employed for the study of effect of temperature were: 95°C (0, 1 day, 2 days, 5 days, 7 days), 85°C (0, 1 day, 4 days, 7 days, 14 days), 72°C (0, 1 week, 2 weeks, 4 weeks, 6 weeks, 8 weeks), 50°C (0, 2 weeks, 4 weeks, 8 weeks, 11 weeks), 37°C (0, 2 weeks, 4 weeks, 8 weeks, 11 weeks) and 4°C (0, 1 week, 4 weeks, 8 weeks, 11 weeks).

Effect of Buffers. RMP-7 was dissolved in buffer at 1 mg/mL, and filtered through a 0.22 μm filter into vials and capped. To limit evaporative losses of water, the vials were stored in closed vessels containing water. Samples were stored at 72°C, and analyzed using HPLC Method F.

Effect of pH. RMP-7 was dissolved in saline at 0.5 mg/mL, adjusted to the desired pH using HCl or NaOH, and then treated as described under Effect of Buffers. The pH was monitored in samples at room temperature. Samples were analyzed using HPLC Method E.

Effect of Temperature. RMP-7 was dissolved in saline at 0.02 mg/mL, adjusted to pH 4.0 using HCl (0.1N), and filtered through a Millex® GV 0.22 μm filter into Wheaton S-265 vials and capped. Samples were stored at 95°C, 85°C, 72°C, 50°C and 37°C, and 4°C. The pH of samples (after reaching room temperature) was monitored over time. Samples were analyzed using HPLC Method A.

Calculations. Purity (%) was defined as 100% × $\text{Area}_{210\text{ for RMP-7}}/\text{Area}_{210\text{ total}}$. Potency was defined as 100% × $\text{Area}_{210\text{ RMP-7 at t}}/\text{Area}_{210\text{ RMP-7 t=0}}$. Purity and potency data were plotted as ln (% purity or potency) versus time, and a degradation rate constant was derived based on first-order kinetics. For the temperature dependence studies, an Arrhenius plot of log k versus 1/T, and an Eyring plot of log (k/T) versus 1/T were constructed. A least squares fit linear regression line for data obtained between 95°C and 50°C was used for projections of rate constants to other temperatures. Calculations were performed for E_a (2.303R(-slope of regression line of Arrhenius plot)), $\Delta H‡$ (2.303R(slope of regression line of Eyring plot)), $\Delta S‡$ (4.576log(k/T) + ($\Delta H‡$/T) − 47.22; log k calculated based on Eyring plot) and $\Delta G‡$ ($\Delta H‡ - T\Delta S‡$), with 75.5°C (348.65 K; the mean value of temperatures used in the Arrhenius plot) used as the temperature for the calculations.

HPLC Equipment and Methods

Three configurations of HPLC equipment were used. System 1: Hewlett Packard 1090. System 2: Applied Biosystems 1000S System, and System 3: Hewlett Packard 1090 Series II.

Method A. Vydac® C_{18} column (250 mm × 4.6 mm). Eluant A = 0.1 M $NaClO_4$, pH 2.5 with H_3PO_4 [85%]; Eluant B = CH_3CN. Gradient: 20% B to 23% B over 6 minutes, 23% B for 8 minutes, 23% to 20% B over 1 minute, and 20% B for 5 minutes; 1.0 mL/min; 210 nm, 214 nm, 230 nm and 280 nm.

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Method B. Vydac® C_{18} column (250 mm × 4.6 mm); Eluant A = 0.1% TFA; Eluant B = CH_3CN. Gradient: 0% B to 40% B over 40 minutes; 1.0 mL/min; 210 nm, 214 nm, 230 nm and 280 nm.

Method C. Vydac® C_{18} column (250 mm × 22 mm). Eluants and Gradient: Same as Method A except: 16 mL/min; 210 nm and 280 nm.

Method D. Vydac® C_{18} column (250 mm × 22 mm) column. Eluants and Gradient: Same as Method B except: 16 mL/min; 210 nm and 280 nm.

Method E. Vydac® C_{18} column (250 mm × 22 mm). Eluant A = 0.1% TFA in water/acetonitrile [86:14]; Eluant B = 0.1% TFA in water/acetonitrile [82:18]. Gradient: 0% B to 100% B over 60 minutes; 16 mL/min; 210 nm and 280 nm.

Method F. Vydac® C_{18} 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: 0% B to 40% B over 40 minutes; 1 mL/min; 210 nm, 214 nm, 230 nm, and 280 nm.

RESULTS AND DISCUSSION

Thermal Degradation Pathways

A representative chromatogram for RMP-7 drug substance stored at 70°C for one day is shown in Figure 1. Two analytical HPLC methods (A and B) were used to increase assurance that the UV absorbing peaks represented single entities. The only difference observed between the 40°C samples and the 70°C samples was the quantity (based on peak area) of each degradant. The sum of the peak areas for the degraded samples was equivalent to the peak area for a standard of RMP-7, indicating mass balance. Four degradants (Degradants A–D) were observed, with Degradant B as the most significant. A comparable degradation pattern was also seen during long term stability studies performed at lower temperatures.

A study of the stability of RMP-7 in aqueous solutions was also performed. In the unbuffered samples no significant changes in pH were observed over the course of the studies. In the buffered aqueous solutions and unbuffered solutions in the pH range 3.5–6.6, Degradants A and B were observed, with Degradant B being the major degradant. In the unbuffered solutions outside of pH 3.5–6.6, Degradants A and B were observed, but were not the major degradation products as numerous small peaks amounted to a greater total peak area.

Data from both pH and buffer studies were analyzed by plotting the natural log of the percent purity versus time. A linear relationship was observed, indicating that the degradation of RMP-7 followed a first order or pseudo-first order pathway. The rate constants are presented in Table I. The optimal stability observed was at a pH of approximately 3.5, with good relative stability observed between pH 3.0 and pH 5.0. Measurement of pH was performed at room temperature, to mimic the situation that would occur on manufacture and stability testing of drug product for clinical trials. The pH stability profile for RMP-7 was comparable to that reported for vasopressin (2).

All observed thermal degradants of RMP-7 drug substance (Degradants A–D) were isolated. Three preparative HPLC methods (C–E) were required to obtain degradants of sufficient purity for structural analysis. Degradant A was isolated using preparative HPLC Method D. Degradants B, C and D were initially separated from the RMP-7 peak using Method C. Degradants C and D were further separated from each other using HPLC method E.

The isolated degradants were characterized by mass spectroscopy (FAB and electrospray), UV spectroscopy, and amino acid analysis. The analytical data are presented in Table II. Most of the sequence of RMP-7 consists of amino acids that can be assessed by amino acid analysis. The C-terminal reduced di-peptide portion of the molecule (4-MeTyrΨ(CH_2NH)Arg) could not be observed in a standard amino acid analysis. Therefore, UV spectral data and molecular weight by mass spectroscopy were required to determine the fate of that portion of the molecule. The proposed structures of degradants and pathways of degradation are presented in Figure 3. An authentic sample of the structure proposed for Degradant C was available (N-Ac^0-RMP-7), and eluted with an equivalent retention time, providing corroborative evidence for the proposed structure of Degradant C. Degradant B was also isolated by HPLC from pooled samples from a study of the unbuffered solutions (pH 3.5–5.0), and its identity verified by mass spectroscopy.

These studies indicated there were two mechanistic pathways active in the thermal degradation of RMP-7 drug substance. One pathway involved the intramolecular cyclization of RMP-7 to form Degradants A (a diketopipera-

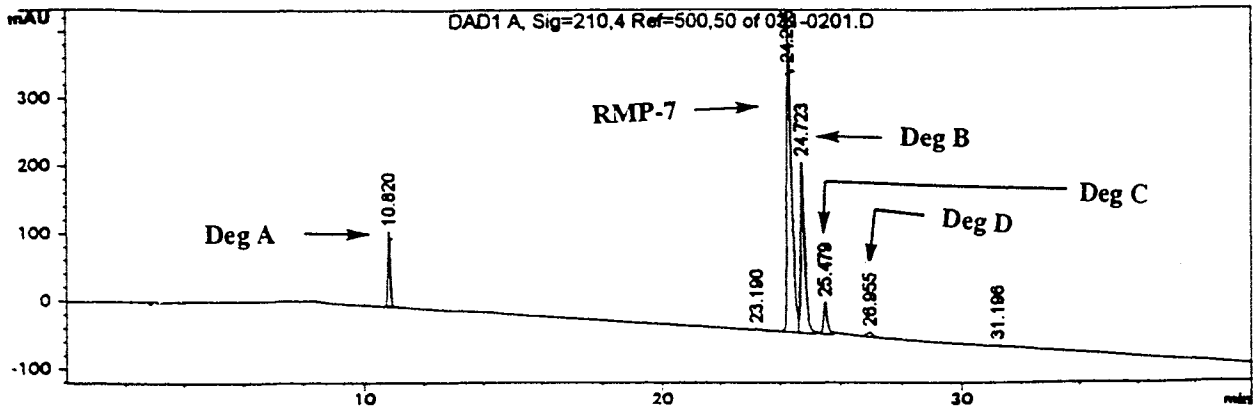

Figure 1: HPLC profile (210 nm detection) showing degradation of RMP-7 drug substance held at 70°C for 1 day (HPLC Method B).

Table I. Summary of Results of the Preformulation Stability Studies Performed at 72°C

RMP-7 Concentration (mg/mL in saline)	pH	Buffer	Rate Constant (h^{-1})
1.0	6.9	20 mM phosphate	6.3×10^{-3}
1.0	5.7	21 mM acetate	4.5×10^{-3}
1.0	4.9	23 mM acetate	2.1×10^{-3}
1.0	5.0	10 mM acetate	1.5×10^{-3}
0.5	2.3	unbuffered	3.2×10^{-3}
0.5	3.0	unbuffered	5.0×10^{-4}
0.5	3.5	unbuffered	3.0×10^{-4}
0.5	4.0	unbuffered	3.7×10^{-4}
0.5	4.5	unbuffered	4.0×10^{-4}
0.5	5.0	unbuffered	5.0×10^{-4}
0.5	5.9	unbuffered	7.1×10^{-4}
0.5	6.6	unbuffered	8.1×10^{-4}
0.5	7.5	unbuffered	1.3×10^{-3}

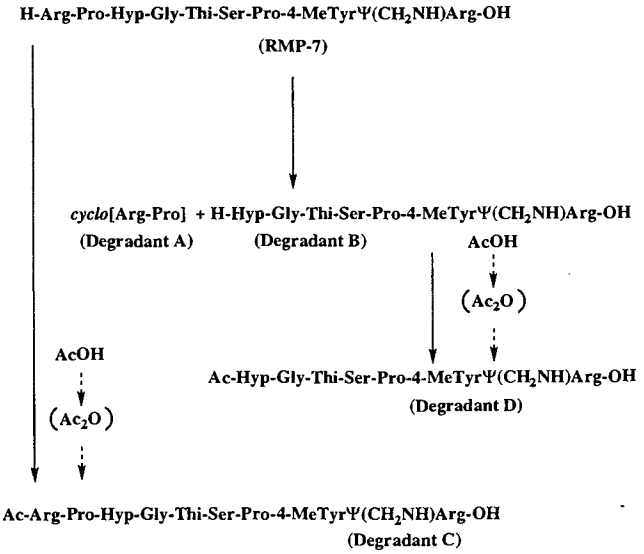

Figure 2: Proposed structures of thermal degradants of RMP-7 drug substance.

zine) and B. The other pathway involved acylation of a free amino group on RMP-7 (and on any Degradant B that had already formed in the sample) by the acetic acid present in RMP-7 drug substance as the counter-ion. This acylation could have proceeded via a condensation of two equivalents of acetic acid, generating actic anhydride, which then functioned as the acylating agent. This acylation resulted in the formation of Degradant C (directly from RMP-7) and Degradant D (via acylation of Degradant B).

Diketopiperazines (such as Degradant A) have been observed as degradation products of other peptides (7–8). Formation of a diketopiperazine occurs more readily when the peptide contains an amino acid (such as proline or glycine) in a position that allows the N-terminal dipeptide to adopt a cis-amide conformation. RMP-7 contains a proline residue at position 2, which facilitates the cyclization reaction that leads to Degradant A (*cyclo*[Arg-Pro]).

This cyclization was also observed in aqueous solutions, and was accelerated in the presence of buffer, as a result of general base catalysis. At very low pH values, the acidity of the solution led to the hydrolysis of amide bonds in RMP-7, making pH values lower than 3 unacceptable. At pH values greater than 4.5, hydroxide ion and the carboxylate anions associated with RMP-7 functioned as base catalysts, which led to decreased stability above pH 4.5. In unbuffered solutions at pH values around 3.5–4.0, the carboxylate of Arg^9 and of the acetate counter-ions were predominantly protonated and unavailable to effect base catalyzed reactions. Also, the pH was not sufficiently low to induce significant acid hydrolysis and not sufficiently high to induce base hydrolysis. These factors contributed to the increased stability seen with the samples at the pH values of 3.5–4.0.

Kinetic Studies-Drug Product

A variety of preclinical studies, and the preformulation work presented here resulted in the selection of a clinical formulation RMP-7 at a concentration of 0.02 mg/mL in unbuffered saline at pH 4.0. A stability study of this formulation at various temperatures (95°C, 85°C, 72°C, 50°C, 37°C and 4°C) was performed to help define storage conditions. Between 50°C and 95°C, the expected temperature dependent degradation was seen (Arrhenius plot seen in Figure 3; $\log k = 8.2169 - 5201.7(1/T)$; k in s^{-1}; $E_a = 23.8$ kcal/mol). An Eyring plot was also performed ($\log (k/T) = 4.8834 - 5052.4\ (1/T)$). Calculated values of $\Delta H‡$, $\Delta S‡$ and $\Delta G‡$ at 75.5°C (the mean value of the temperatures used to generate the Arrhenius plot) were 23.1 kcal/mol, −24.9 eu, and 31.8 kcal/mol, respectively. At temperatures of 37°C and 4°C, time-points later than 0 were statistically different from the 0 time-point (2–3% loss of potency with no accompanying degradation observed), but such later time-points were not significantly different from each other. The initial loss in

Table II. Analytical Data on Degradants

Degradant	Molecular Weight	UV Spectra 280 nm[a]	Amino Acid Analysis	RRT[b] System A	RRT[b] System B
A	253	no	Arg, Pro	0.26	0.45
B	844	yes	Hyp, Gly, Thi, Ser, Pro	0.92	1.02
C	1140	yes	Arg, Pro (2), Hyp, Gly, Thi, Ser	0.92	1.05
D	886	yes	Arg, Pro (2), Hyp, Gly, Thi, Ser	0.92	1.11

[a] A λ_{max} observed near 280 nm indicates the presence of Tyr(Me).
[b] RRT, the relative retention time, is the ratio of the retention time of the peak of interest to the retention time of intact RMP-7.

potency of the samples stored at the low temperatures indicated that some RMP-7 adsorbed to the glass surface of the vial. The tendency of RMP-7 to adsorb to glass was confirmed using ^{3}H-RMP-7 (data not shown). RMP-7 drug product is currently being stored 2°C–8°C. Using the Arrhenius equation derived from the data measured between 95°C and 50°C, a rate constant of 5.19×10^{-11} s^{-1} can be calculated for 8°C storage. Long term stability studies have shown that the drug product formulated as described above is stable at 2°C–8°C for at least 2 years.

In summary, the results presented have shown two major pathways for degradation of RMP-7. A portion of RMP-7 drug substance is transformed into an acetylated adduct presumably by acylation directly or indirectly from the acetate counterion, which indicates that the choice of counterion can affect stability. The predominant degradation pathway for drug substance and a number of aqueous solutions involves the formation of an N-terminal diketopiperazine and a C-terminal heptapeptide, which is a consequence of the sequence of RMP-7 (like bradykinin) containing a proline at position 2. Such a cyclization can be minimized in solution by avoiding general base catalysts such as phosphate or acetate buffers.

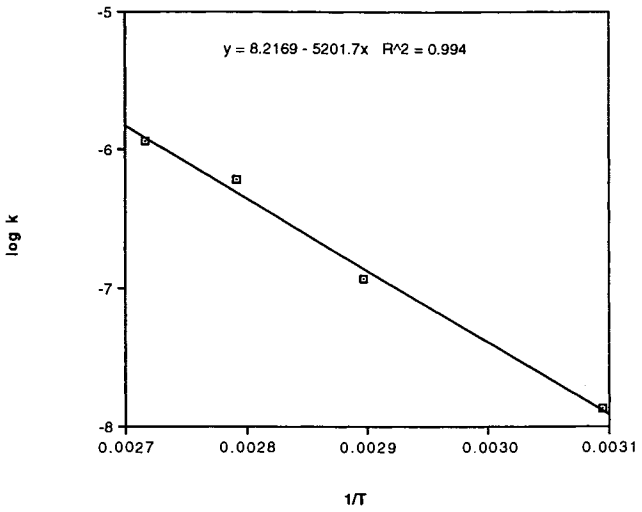

Figure 3: Stability of RMP-7 drug product (0.02 mg/mL in unbuffered saline at pH 4.0) as a function of temperature (Arrhenius plot of log rate constant vs 1/T). Values for k are expressed in s^{-1}.

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