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A competitive chemiluminescent enzyme-linked immunosorbent assay for the determination of RMP-7 in human blood[1]

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Abstract

RMP-7, a bradykinin agonist, is a synthetic nonapeptide designed to enhance the delivery of therapeutics to the central nervous system. A sensitive, competitive chemiluminescent enzyme-linked immunosorbent assay (ELISA) for quantifying RMP-7 in human blood samples has been developed. Rabbit antibodies against RMP-7 were produced using the conjugate of RMP-7 to keyhole limpet hemocyanin through glutaraldehyde. Biotinylated RMP-7, conjugated via *N*-hydroxysuccinimide ester, was used as the tracer. A premixed solution of biotinylated alkaline phosphatase and avidin was used to quantify the tracer, with a dioxetane-based compound as the chemiluminescent substrate. The method involves treating blood samples with organic solvents to precipitate proteins, evaporating the supernatants to dryness, reconstituting residues in PBS and assaying the buffer solutions with the ELISA. The assay, using 1.0 ml of whole blood, has precision and accuracy within $\pm$ 20% over the concentration range 25–800 pg ml^{-1}. There are no significant endogenous interferences. The assay has been successfully used to support clinical trials of RMP-7.

Keywords: Bradykinin agonist; Competitive chemiluminescent ELISA

1. Introduction

Synthetic analogs of naturally occurring peptides are of increasing interest in the development of novel therapeutic agents [1]. Many of these analogs have been modified to enhance the potency and specificity of the endogenous peptides, and to prolong their half-life in the body. Since these compounds are highly potent and usually administered in minute amounts, their concentration in body fluids is often in the nanogram per milliliter (ng ml^{-1}) to picogram per milliliter (pg ml^{-1}) range. Accurate and specific measurement of these compounds and their metabolites at such

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H-Arg^1-Pro^2-Hyp^3-Gly^4-Thi^5-Ser^6-Pro^7-Tyr(Me)$^8\Psi$(CH$_2$NH)-Arg^9-OH

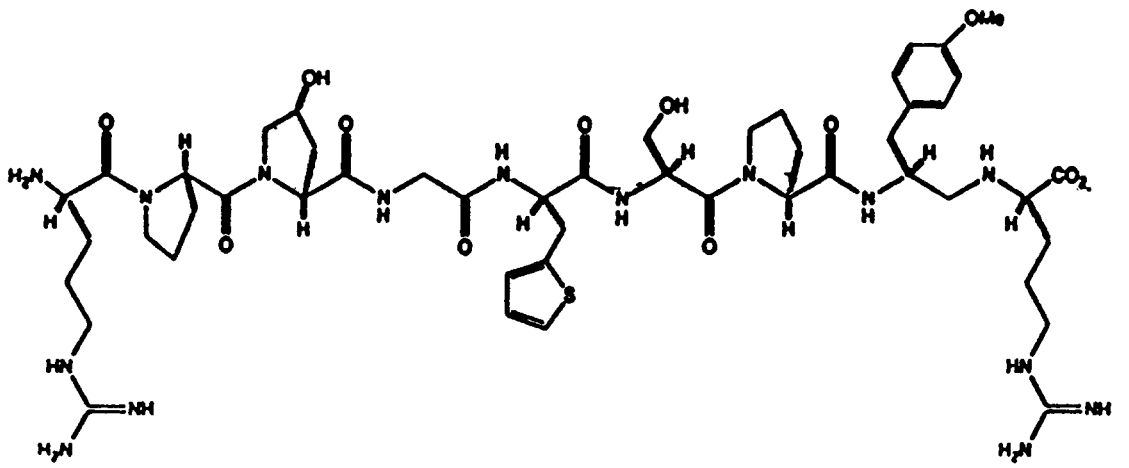

Fig. 1. Structure of RMP-7, an analog of bradykinin.

levels in biological fluids, in support of pharmacokinetic (PK) and metabolism studies, presents a significant analytical challenge. Radioimmunoassay (RIA), despite frequent concerns over its specificity, has been the most commonly used method for these determinations, chiefly because of its high sensitivity [2–6]. HPLC, using either pre- or post-column fluorescence derivatization has been extensively exploited in recent years as an alternative approach [7–16]. HPLC coupled with mass spectrometric detectors has also been used [17–20].

RMP-17 is a synthetic analog of bradykinin (Fig. 1). It contains two amino acids not normally found in mammalian systems (thienylalanine and 4-methyltyrosine) and a reduced peptide bond (Ψ–CH_2–NH) located between Tyr(Me)^8 and Arg^9. The compound is being developed to provide a method for enhancing the permeability of the blood–brain barrier (BBB) and to allow increased access of therapeutic agents to the central nervous system (CNS), thereby improving efficacy in the treatment of CNS disorders. RMP-7 temporarily increases the permeability of the BBB by an apparent interaction with bradykinin B2 receptors on the endothelial cells which comprise the BBB [21–23]. In animal models, RMP-7 increases brain uptake of various compounds, which normally have limited ability in crossing the BBB [24–26]. Its maximum dose without causing significant side-effects in humans is $\approx$ 300 ng kg^{-1} by intravenous administration, which gives an estimated maximum plasma concentration in the low ng ml^{-1} range. Therefore, clinical PK studies of RMP-7 require an analytical method with a limit of quantification in the pg ml^{-1} range.

In this report, a sensitive competitive enzyme-linked immunosorbent assay (ELISA) for the determination of RMP-7 in whole human blood is described. Rabbit antibodies against RMP-7 were produced using the conjugate of RMP-7 to key-hole limpet hemocyanin. Biotinylated RMP-7 was used as the tracer. A premixed solution of biotinylated alkaline phosphatase and avidin, referred to as ABS reagent, was used to quantify the tracer, with a dioxetane-based compound as the chemiluminescent substrate. The method involves treating blood samples with organic solvents to precipitate proteins, evaporating the supernatants to dryness, reconstituting residues in PBS and assaying the buffer solutions with the ELISA. The assay, using 1.0 ml of blood, has precision and accuracy of $\approx \pm 20\%$ over the concentration range 25–800 pg

ml^{-1}. There are no significant endogenous interferences. The assay has been used to support clinical trials of RMP-7.

2. Experimental

2.1. Chemicals and reagents

N-hydroxysulfosuccimide ester of biotin (NHS-biotin) was obtained from Calbiochem (La Jolla, CA). RMP-7 was manufactured by Peninsula Inc. (Belmont, CA) for Alkermes. Keyhole limpet hemocyanin (KLH) and glutaraldehyde were purchased from Sigma Chemical Co. (St. Louis, MO). The prepacked ImmunoPure Immobilized Protein A AffinityPak columns (cat. # 20356), horseradish peroxidase (HRP), alkaline phosphatase (AP), HRP-labeled goat anti-rabbit antibodies, the substrates tetramethylbenzidine (TMB), azinobis (3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) and *p*-nitrophenyl phosphate (PNPP), and SuperBlock solution were all from Pierce Chemical Co. (Rockford, IL). The chemiluminescent reagents, including the substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3,3,1,1]decan}-4-yl)phenyl phosphate (CSPD) and Sapphire (enhancer), were from Tropix (Bedford, MA). The ABC reagents were purchased from Vector Laboratories, Inc. (Burlingame, CA). Tween-20 (EIA grade) was from Bio-Rad. Pooled whole human blood was obtained from American Red Cross (Boston, MA). All other reagents were of analytical grade. Solutions were prepared in distilled and deionized water.

2.2. Apparatus

Colorimetric ELISAs were carried out in 96-well plates (Corning, NY) and the absorbance was read with a MR7000 microplate reader from DynaTech (Chantilly, VA). The chemiluminescent ELISAs were carried out using white and opaque 96-well plates from Dynatech (Microlite 1, flat bottom, cat. # 011-010-7416). The chemiluminescent emission was read with a DynaTech ML1000 luminometer and the data were captured on a Macintosh IICi computer using Lum2BioCalc (version 0.5) software from DynaTech.

2.3. Production of Polyclonal Antibodies

The immunogen was prepared by conjugating RMP-7, through its α-NH_2 group, to KLH using glutaraldehyde as the linker according to the method described by Zegers et al. [27]. The immunization of eight female New Zealand White rabbits with the immunogen was made using a standard protocol [28]. Briefly, the rabbits were initially immunized intradermally at three sites, using 100 μl of the conjugate in Freund's Complete Adjuvant (FCA) at each site. On days 14, 21, 42 and 83, the animals were given booster immunizations with the conjugate in Freund's Incomplete Adjuvant (FIA) intramuscularly. One week after the last immunization, the rabbits were bled from the ear vein and the antibody titers were determined.

Antibody titers of the antisera were determined by a colorimetric ELISA. Microtiter plates were coated with RMP-7 by adding 100 μl of 10 $\mu g\ ml^{-1}$ RMP-7 in coating buffer (i.e. 0.1 M carbonate buffer, pH 9.6). The plates were covered and incubated at room temperature for 2 h. After washing with 0.05 M PBS containing 0.05% Tween-20 (washing buffer), 100 μl of diluted rabbit antisera was added to each well and incubated for 1 h at room temperature. After washing with the washing buffer, 100 μl of HRP-conjugated goat anti-rabbit diluted 1000-fold with the PBS containing 0.2% BSA was added to each well and incubated for 1 h at room temperature. Following another washing step with the washing buffer, 100 μl of ABTS was added to each well. After 20 min of incubation at room temperature, the plates were read at 410–650 nm.

The immunoglobulin G (IgG) fraction of the antiserum with the highest titer was purified using Protein-A columns following the procedures recommended by the manufacturer. The IgG concentration in the final solution was measured by its absorption at 280 nm.

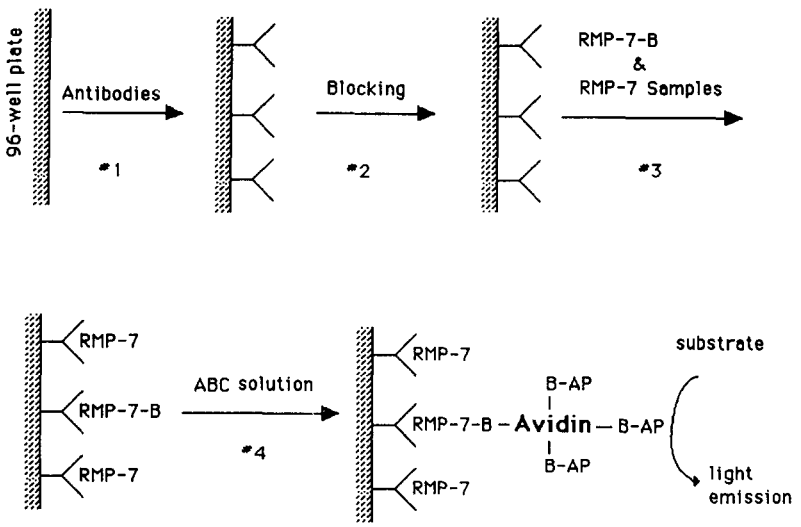

Fig. 2. The competitive chemiluminescent ELISA for RMP-7.

2.4. Biotinylation of RMP-7

NHS-biotin (12.5 mg) dissolved in 50 μl of DMSO was added to 1.0 ml of RMP-7 solution (10 mg ml^{-1}) in 10 mM HEPES (pH 8.5). The solution was incubated for 24 h at room temperature. Trifluoroacetic acid (TFA) solution (1.0 ml of 0.1% aqueous) was added to it, followed by application of the solution to a pre-washed Sep-Pak C18 cartridge. The cartridge was washed with 5 ml of the TFA solution and 5 ml of the TFA solution/acetonitrile (95:5, v/v). The cartridge was eluted with 5 ml of the TFA solution/acetonitrile (60:40, v/v). The eluent was collected, lyophilized and stored at −80°C. Biotinylated RMP-7 solution (0.5 mg ml^{-1}) was prepared in water for use in the ELISA. The solution was stored at 2–8°C for no more than 3 months.

2.5. Collection and treatment of blood samples

During the Phase I clinical trial of RMP-7, healthy male volunteers received single and escalating doses of RMP-7 (intravenous bolus) with a maximum dose of 300 ng kg^{-1}. Blood samples ($\approx$4 ml) were collected from each of the volunteers prior to, and at various time points after, intravenous administration of RMP-7. They were immediately quenched after collection to stop possible degradation of RMP-7 in the blood by adding 6 ml of ethanol and 120 μl of 1.0 M HCl. The samples were then centrifuged to remove solid materials and the supernatants were stored at −80°C until analysis.

On the day of analysis, the samples were allowed to thaw to room temperature and centrifuged at 3000*g* for 5 min. 2 ml portions of supernatant from each of the samples were pipetted into clean, labeled test tubes. 2 ml of acetonitrile was added to further precipitate the proteins. The samples were centrifuged at 3000*g* for 10 min to remove the precipitate. The supernatants were evaporated to dryness under a stream of nitrogen at 40°C and reconstituted in 1.0 ml 0.05 M PBS. Samples with concentrations greater than the highest standard were re-assayed by reconstituting the residues in 5.0 ml 0.05 M PBS. The solutions were assayed using the ELISA as described below.

2.6. ELISA procedure

The major steps involved in the competitive ELISA are schematically illustrated in Fig. 2. The anti-RMP-7 IgG was diluted in the coating buffer to a concentration of 2 μg ml^{-1} and was coated onto a 96-well plate by incubating 100 μl per well at room temperature on an orbital shaker for 2 h.

The plate was washed once with the washing buffer. The plate was blocked by incubating 300 μl of SuperBlock solution per well for 15 min at room temperature on the orbital shaker. The plate was washed three times with the washing buffer. Pretreated RMP-7 standards, controls and unknown samples (50 μl per well) were added to the plate in triplicate and were incubated at room temperature for 1.0 h. Following addition of 50 μl of biotinylated RMP-7 solution, the plate was incubated at room temperature for 1 h and washed three times. Avidin/biotin-AP solution (ABC reagent, 100 μl per well) was added to the plate and was incubated at room temperature for 1 h. The plate was washed five times with the washing buffer. The substrate (CSPD) solution (50 μl per well) was added to the plate and was incubated for 30 min. The plate was read on the luminometer.

2.7. Data analysis

Standard curves were constructed by plotting the observed emission signals against the concentrations in the standards. The data were then fitted into a four-parameter equation (shown below) using non-weighted least-squares regression analysis:

$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^b}$$

where y is the signal, x is the concentration of RMP-7, and a, b, c and d are constants. Goodness of the fit was evaluated by back-calculating the concentrations of the standards using the best-fit equation and comparing them with their theoretical values. The concentrations of spiked and unknown samples were calculated from the equation.

3. Results and discussion

RIA has been almost exclusively used for the determination of bradykinin in biological fluids with I^{125} as the tracer [29]. However, the iodination of RMP-7 is not straightforward because of its lack of a tyrosine group, and moreover I^{125} has a short shelf-life. Therefore, the enzymes, HRP and AP, were used as the alternative tracers. Because of the small size of RMP-7 (WM ≈ 1100 Da), a competitive assay was chosen as the format with the specific IgG antibodies coated onto a 96-well microtiter plate. Initial experiments with colorimetric substrates, TMB and ABTS for HRP and PNPP for AP, showed relatively high background and a sensitivity in the low ng ml^{-1} range, which was not acceptable for the determination. Subsequently, a dioxetane-based chemiluminescent substrate (CSPD) was used with AP as the tracer, which resulted in an assay with a sensitivity in the ≈ 100 pg ml^{-1} range in blood samples. The assay was then employed for quantifying RMP-7 in some of the blood samples from the highest dose group (300 ng kg^{-1}). After examining the data, it was concluded that the sensitivity of the assay needed further improvement in order to obtain meaningful PK data from the blood samples. Biotin was consequently used as the tracer and a commercial ABC kit with biotinylated AP was used to further amplify the signal prior to the addition of the chemiluminescent substrate. After initial optimization, the assay was capable of quantifying RMP-7 down to 25 pg ml^{-1} in whole blood with a measurement error of ≈ ± 20%.

3.1. Immunization and antibody production

RMP-7 linked to KLH was used to immunize eight rabbits, which then produced detectable antibodies to RMP-7 after several booster injections. The antibody titers of the rabbits reached plateau values after ≈ 3 months of immunization. One rabbit (# 1A) produced antiserum with the highest titer with a value of ≈ 5000–10 000 (Fig. 3). The serum was purified using protein-A columns to isolate the IgG fraction. The concentration of the IgG antibodies in the purified solution was determined to be ≈ 1 mg ml^{-1} based on an absorption coefficient of 1.4 AU mg^{-1} of protein at 280 nm. The solution was pipetted into a series of 200 μl aliquots, stored at − 80°C and used in all subsequent evaluations and assays.

3.2. The chemiluminescent detection

The use of chemiluminescent detection in immunoassay has become increasingly popular in recent years because of the high sensitivity and wide dynamic range afforded by this method [30]. The three most commonly used chemiluminescent reactions are: the oxidation of acyl hydrazides (luminol), acridinium esters and trichlorophenyl oxalate esters in the presence of a catalyst such as HRP. They all require the controlled addition of hydrogen peroxide, alkali and other reagents. A new class of compounds have been synthesized which contain 1,2-dioxetane [31]. In the presence of AP, the compounds hydrolyze to produce a four-member cyclic peroxide that is metastable at room temperature in water. It decomposes into a carbonyl in an excited and long-lived triplet state, which emits light as it decays to its ground state. The dioxetane-based compounds have the advantages that they are rather specific to AP and do not need any other reagents to generate light in aqueous solutions. The emission has a relatively long half-life. The intensity of the emission can be further enhanced by adding quaternary ammonium polymers.

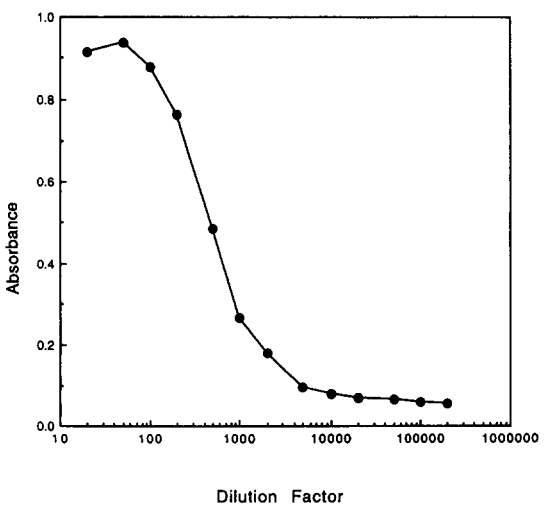

Fig. 3. Titer of the antiserum by ELISA.

3.3. Assay optimization

The optimal conditions for competitive assays are such that $\approx 50\%$ of the tracer is bound to the antibodies for a "blank" sample [32–34]. Under this condition, the assay has the most desirable analytical characteristics: high sensitivity and wide dynamic concentration range. The sensitivity of a competitive assay usually increases with decreasing concentrations of the tracer and the antibodies, but its dynamic concentration range decreases at the same time. In order to develop an assay with a high sensitivity and a reasonably wide dynamic range, the concentrations of the tracer and the antibodies are usually optimized experimentally. This is achieved either by titrating the antibodies with the tracer or by titrating the tracer with the antibodies. In this study, the IgG antibodies were titrated with the tracer.

The titration of the antibodies was carried out by coating the wells on a plate with increasing concentrations of the antibodies. Biotinylated RMP-7 (100 μl) at a given concentration was then added to all the wells. The relative amount of biotinylated RMP-7 bound in each of the wells was determined by measuring the emission signal after adding the substrate. The signal was plotted against the concentration of the IgG antibodies to obtain the titration curve. A family of titration curves at several different concentrations of biotinylated RMP-7 is shown in Fig. 4. The signal intensity from the bound biotinylated RMP-7 first increases and then reaches a plateau value with increasing antibody concentrations. The plateau value decreases with decreasing concentrations of biotinylated RMP-7.

The optimal concentration of the biotinylated RMP-7 was chosen to be 5 ng ml^{-1} and that of the IgG antibodies was chosen to be 1 μg ml^{-1}, which gives an initial percentage binding of the tracer of $\approx 50\%$. Standard curves for RMP-7 run under this condition in PBS gave a dynamic concentration range of $\approx 1-1000$ pg ml^{-1}.

3.4. Specificity

The specificity of a competitive immunoassay depends very much upon the antibodies' cross-re-

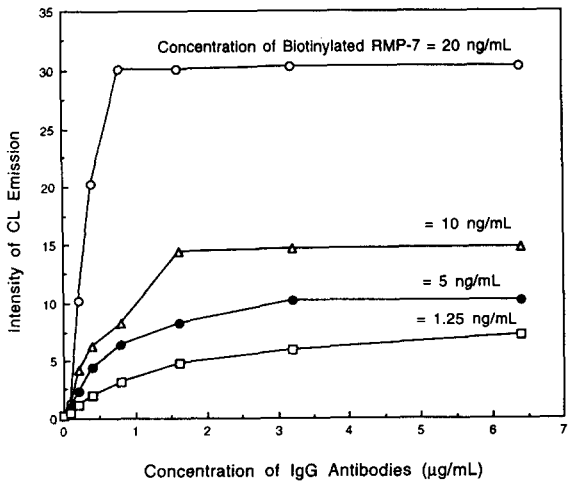

Fig. 4. Titration curves of the IgG antibodies at four different concentrations of biotinylated RMP-7.

activity to other components in the sample matrix. Since RMP-7 is an analog of bradykinin and the endogenous level of bradykinin in the blood stream can be as high as 5 ng ml^{-1} [26], the antibodies' cross-reactivity to bradykinin was examined by determining the response curves of the ELISA to RMP-7 and bradykinin in PBS, as shown in Fig. 5. B/B_0 is the percentage of biotinylated RMP-7 bound, which can be calculated by dividing the corrected signal value (observed value minus the value of nonspecific binding) at a particular concentration by that of the "blank". As can be seen, the interference from bradykinin is minimal.

3.5. Sample preparation and recovery

Studies using tritium-labeled RMP-7 (^{3}H-RMP-7) and HPLC coupled with a radioactive detector (HPLC/RAD) showed that a significant amount of RMP-7 was degraded during the sample collection process if plasma or serum were collected following standard protocols in a clinical setting. This phenomenon has been previously observed for bradykinin and other polypeptides [29] because of these compounds' extreme susceptibility to degradation by proteolytic enzymes found in biological matrices. Several quenching procedures have been proposed to instantaneously inhibit the enzymes in the blood, such as adding organic solvents, acids and enzyme inhibitors, heating the sample to 100°C, and using charcoal or other resins to quickly adsorb and separate the polypeptides from the matrix. Each of the methods has its pros and cons. The procedure chosen here involved adding ethanol annd hydrochloric acid to the blood sample immediately after its collection. Studies with ^{3}H-RMP-7 demonstrated that, using this protocol, the degradation of RMP-7 was minimal. Experiments also showed that, when the quenching procedure was followed closely, 1.0 ml of blood sample would generate $\approx$ 2.0 ml of supernatant. Consequently, 2.0 ml of the supernatant was always taken for assaying the unknown samples.

The absolute recovery of RMP-7 from the blood samples, following the quenching procedure and further protein precipitation with acetonitrile, was determined by measuring the recovery of radioactivity from ^{3}H-RMP-7-spiked whole blood. It was $\approx$ 65% when the spiked concentration of ^{3}H-RMP-7 was $\approx$ 10 ng ml^{-1}. The loss was mostly due to the co-precipitation of RMP-7 during the quenching step.

3.6. Standard curves and sensitivity

In order to compensate for the significant loss of RMP-7 and the variability of the loss with

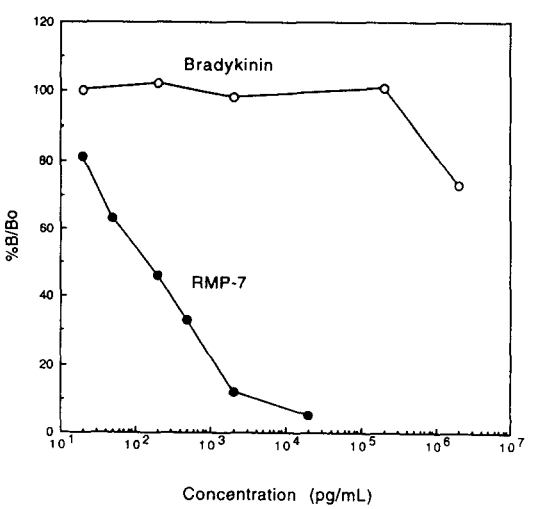

Fig. 5. The response curves of the ELISA to bradykinin and RMP-7.

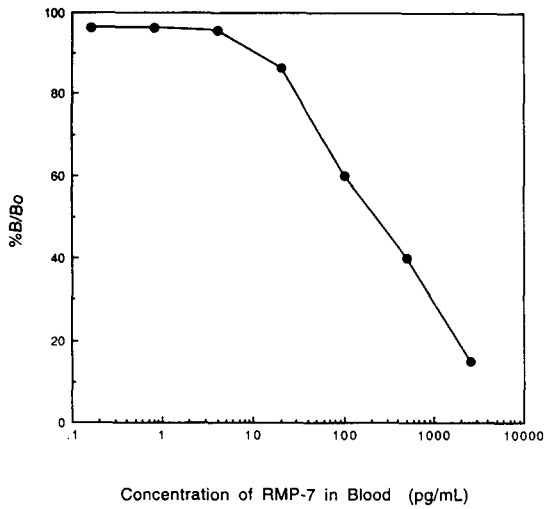

Fig. 6. A plot of $\%B/B_0$ versus concentration of RMP-7 in whole blood.

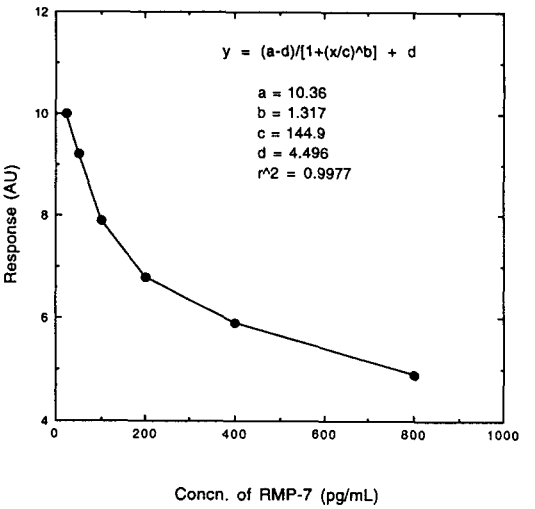

Fig. 7. A typical standard curve of the ELISA for RMP-7 in whole blood. The data are fitted into a four-parameter equation.

concentration of RMP-7 from the quenching procedure, standards were prepared in pooled blood. A plot of $\%B/B_0$ versus concentration of RMP-7 in the blood is shown in Fig. 6. The limit of detection of the assay, defined as the concentration which gives a signal twice the standard deviation of the blank, was ≈ 10 pg ml^{-1}. The upper limit of the assay's dynamic range was > 800 pg ml^{-1}. The concentration of RMP-7 was ≈ 250 pg ml^{-1} at the point of $B/B_0 = 50\%$.

For the validation of precision and accuracy, and routine analysis of the blood samples, six standards covering the concentration range 25–800 pg ml^{-1} were run with each plate along with controls and unknown samples. Fig. 7 shows a typical standard curve. Eight standard curves were run on different days over a period of 2 weeks. There was considerable variation ($\approx 30\%$ relative standard deviations) in the values of a, b, c and d among different plates. For a specific plate, however, the correlation coefficient (r^2) of the standard curve was always > 0.99. The back-calculated values agreed within $\pm$ 15% with the theoretical values of the standards.

3.7. Precision and accuracy

Control samples at each of the three spiked concentrations (50, 100 and 400 pg ml^{-1}) were analyzed for eight replicates on each plate and on eight separate days. The results are presented in Table 1. The method showed good precision and accuracy with intra-assay relative standard deviation (RSD) of 16.1% and inter-day RSD of 13.7%. The relative recovery ranges from 99.5% to 110.3%. The effects of repeated freezing/thawing were also investigated. After three cycles, there was no significant change observed.

4. Conclusion

The competitive chemiluminescent ELISA has been successfully applied to the determination of

Table 1
Precision and accuracy of the assay

Concentration (pg ml^{-1})			%RSD	
Added	Measured[a]	%Recovery[a]	Intra-assay[b]	Inter-assay[a]
50	49.8	99.5	15.5	15.8
100	110.3	110.3	17.4	12.9
400	407.5	101.9	15.5	12.3
	Average:	103.9	16.1	13.7

[a] $n = 8 \times 8 = 64$.
[b] $n = 8$.

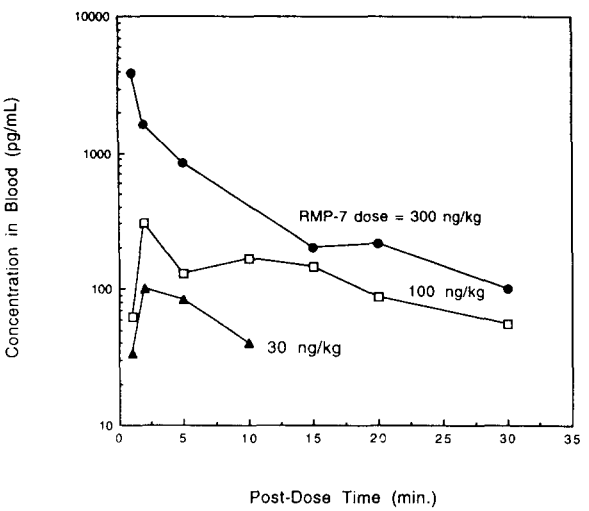

Fig. 8. Concentrations of RMP-7 in blood for three male volunteers at various time points after they received intravenous doses (30, 100 and 300 ng kg^{-1}) of RMP-7.

RMP-7 in human blood samples in support of its Phase I trial. Fig. 8 shows the blood concentration–time curves of three male volunteers following intravenous administration of RMP-7 at three different doses. For the highest dose of 300 ng kg^{-1}, the assay was able to quantify RMP-7 through at least four half-lives from its maximum concentration. For the doses of 100 and 30 ng kg^{-1}, the assay was still capable of giving a general trend of the PK profiles.

In conclusion, a sensitive, specific and accurate ELISA has been developed for the determination of a nonapeptide (RMP-7) in whole human blood. The high sensitivity of the method has allowed it to be employed to study the PK characteristics of RMP-7 in humans within a dose range of interest for this clinical program.

References

[1] M.D. Taylor and G.L. Amidon (Eds.), Peptide-Based Drug Design: Controlling Transport and Metabolism, American Chemical Society, Washington, DC, 1995.
[2] J.F. Rehfeld, Trends Anal. Chem., 8 (1989) 102–106.
[3] I. Yamazaki and H. Okada, Endocrinol, Jpn., 27 (1985) 593–605.
[4] A.S. Yuan, E.L. Hand, M. Hichens, T.V. Olah, A. Barrish, C. Fernandez-Metzler and J.D. Gilbert, J. Pharm. Biomed. Anal., 11 (1993) 427–434.
[5] S. Chishima, J. Pharm. Biomed. Anal., 12 (1994) 795–804.
[6] S. Lundin, P. Melin and H. Vilhardt, Acta Endocrinol., 108 (1985) 179–183.
[7] C. Miller-Stein, B.Y.-H. Hwang, G.R. Rhodes and V.K. Boppana, J. Chromatogr., 631 (1993) 233–240.
[8] V.K. Boppana and G.R. Rhodes, J. Chromatogr., 507 (1990) 79–84.
[9] G.R. Rhodes and V.K. Boppana, J. Chromatogr., 444 (1988) 123–131.
[10] K.J. Dave, J.F. Stobaugh, T.M. Rossi and C.M. Riley, J. Pharm. Biomed. Anal., 10 (1992) 965–977.
[11] M. Kai, J. Ishida and Y. Ohkura, J. Chromatogr., 430 (1988) 271–278.
[12] H. Koning, H. Wolf, K. Venema and J. Korf, J. Chromatogr. Biomed. Appl., 533 (1990) 171–178.
[13] M. Ohno, M. Kai and Y. Ohkura, J. Chromatogr., 392 (1987) 309–316.
[14] M. Kai, T. Miyazaki, Y. Sakamoto and Y. Ohkura, J. Chromatogr., 322 (1985) 473–477.
[15] M. Kai, T. Miura, J. Ishida and Y. Ohkura, J. Chromatogr., 345 (1985) 259–265.
[16] M. Ohno, M. Kai and Y. Ohkura, J. Chromatogr., 490 (1989) 301–310.
[17] J. Crowther, V. Adusumalli, T. Mukherjee, K. Jordan, P. Abuaf, N. Corkum, G. Goldstein and J. Tolan, Anal. Chem., 66 (1994) 2356–1361.
[18] R.B. van Breemen and R.G. Davis, Anal. Chem., 64 (1992) 2233–2237.
[19] J.J. Kusmierz, R. Sumrada and D.M. Desiderio, Anal. Chem., 62 (1990) 2395–2400.
[20] E. Gelpi, J. Chromatogr., 703 (1995) 59–80.
[21] S.R. Doctrow, S.M. Abelleira, L.A. Curry, R. Heller-Harrison, J.W. Kozarich, B. Malfroy, L.A. McCarroll, K.G. Morgan, A.R. Morrow, G.F. Musso, J.A. Straub and C.A. Gloff, J. Pharmacol. Exp. Ther., 271 (1994) 229–237.
[22] R.T. Bartus, P.J. Elliott, N.J. Hayward, R. Dean, K. Matsukado, K.L. Black and S. Fisher, Immunopharmacology, 33 (1996) 270–278.
[23] E. Sanovich, R.T. Bartus, P.M. Friden, R. Dean, H. Le and M. Brightman, Brain Res., 705 (1995) 125–135.
[24] N.J. Hayward, P.J. Elliott, M.R. Huff, T.L. Nagle, K. Matsukado, K.L. Black and R.T. Bartus, J. Neurosurg., (1996) in press.
[25] T. Inamura, T. Nomura, R.T. Bartus and K.L. Black, J. Neurosurg., 81 (1994) 752–758.
[26] P.J. Elliott, N.J. Hayward, R.L. Dean and R.T. Bartus, Immunopharmacology, 33 (1996) 205–208.
[27] N. Zegers, K. Gerritse, C. Deen, W. Boersma and E. Classen, J. Immunol. Methods, 130 (1990) 195–200.
[28] F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (Eds.), Current

Protocols in Molecular Biology, Vol. 2, John Wiley, New York, 1990, Chapter 11.
[29] T.L. Goodfriend and C.E. Odya, in B.M. Jaffe and H.R. Behrman (Eds.), Methods of Hormone Radioimmunoassay, 2nd edn., Academic Press, New York, 1979, Chapter 46.
[30] I. Weeks and J.S. Woodhead, Trends Anal. Chem., 7 (1988) 55–58.
[31] I. Bronstein and P. McGrath, Nature, 338 (1989) 599–600.
[32] D.W. Chan (Ed.), Immunoassay: A Practical Guide, Academic Press, San Diego, CA, 1987.
[33] D. Wild, Immunoassay Handbook: Products, Applications, Principles, Stockton, New York, 1994, Chapter 2.
[34] R.P. Ekins, in W.P. Collins (Ed.), Alternative Immunoassays, Wiley, New York, 1985, pp. 219–237.