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International Journal of Pharmaceutics 353 (2008) 56–64
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Single- and multiple-dose pharmacokinetics of exendin-4 in rhesus monkeys

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Received 20 June 2007; received in revised form 16 October 2007; accepted 10 November 2007
Available online 17 November 2007

Abstract

A radioimmunoassay (RIA) for the measurement of exendin-4 concentration in rhesus monkeys serum was developed and validated. The radioimmunoassay described here was sensitive, linear, accurate, precise, and reproducible. Range of the assay was 25–2000 pg/ml. Using this method we characterized the pharmacokinetics and accumulation of exendin-4 in rhesus monkeys. Following s.c. administration at doses rate of 1, 3 and 10 μg/kg, average C_{max} ranged from 2.26 ± 0.15 to 22.72 ± 1.54 ng/ml, and $AUC_{0-\infty}$ ranged from 3.43 ± 0.05 to 47.1 ± 0.10 ng h/ml. As compared to the i.v. administration at a single dose of 3 μg/kg, the absolute bioavailability after s.c. administration were estimated to be 67.3 ± 5.3, 75.1 ± 4.7 and 72.7 ± 8.4% for 1, 3 and 10 μg/kg dose, respectively. After daily s.c. administration at 10 μg/kg for 7 consecutive days, the accumulation ratio was approximately to 1.0, indicating no accumulation upon multiple doses in the monkeys.

Keywords: Radioimmunoassay; Magnetic beads; Exendin-4; Pharmacokinetics; Rhesus monkeys

1. Introduction

Diabetes mellitus (DM) has reached epidemic proportions and numerous reports have documented the sharply increasing incidence of DM in the world (Zimmet et al., 2001; Lenhard and Gottschalk, 2002; Wild et al., 2004). In 2003, the International Diabetes Federation (IDF) estimated that almost 200 million people around the world had DM. By 2025 this figure is expected to rise to 333 million, amounting to 6.3% of the world's population living with DM. Approximately 90–95% of people with DM have type 2 diabetes mellitus (T2DM), which is characterized by hyperglycemia, insulin resistance, absolute or relative insulin deficiency, hyperglucagonemia and increased hepatic glucose production (Gadsby, 2002; Knowler et al., 2002; Leahy, 2005). Control of circulating glucose levels is rarely optimal, and many currently available therapies also have unfavorable side effects and restrictions, limiting the extent of their use (DeFronzo, 2004). Thus, there is an imperative need for novel therapeutic approaches for glycemic control that can complement existing therapies and possibly attempt to preserve normal physiological response to meal intake. One such approach is based on the action of the glucagon-like peptide 1 (GLP-1). GLP-1 is an incretin hormone, which is released from the L cells of the distal intestine in response to nutrients. It possesses a number of beneficial antidiabetic properties, such as glucose-dependent enhancement of insulin secretion, glucose-dependent suppression of inappropriately high glucagon secretion, slowing of gastric emptying, reduction of food intake and body weight. It may even promote β-cell preservation and improved neogenesis (Perry and Greig, 2003; Takei and Kasatani, 2004; Arulmozhia and Portha, 2006). Unfortunately, the usefulness of the native peptide is limited by its metabolic instability. Circulating GLP-1 undergoes rapid proteolytic cleavage by dipeptidyl peptidase-IV (DPP-IV), a ubiquitous serine protease, and biologically active GLP-1 has an apparent serum half-life of only 1–2 min in humans (Deacon

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doi:10.1016/j.ijpharm.2007.11.016

et al., 1998). Given the rapid inactivation and short half-life of GLP-1, long acting agonists of the GLP-1 receptor have been developed.

Exendin-4, a 39-amino acid peptide that shares 53% amino acid sequence homology with GLP-1, was originally extracted from the venom of the *Heloderma suspectum* lizard monster (Eng et al., 1992; Goke et al., 1993). Exendin-4 has been shown in a variety of animal and cell models as well as in humans to share the same glucoregulatory actions of GLP-1 (Edwards et al., 2001; Egan et al., 2002; Meneilly et al., 2003; Nielsen et al., 2004; Michael and Juris, 2005; Iltz et al., 2006). Moreover, the actions of exendin-4 were also potentiated by its resistance to cleavage by DPP-IV, resulting in a longer half-life and duration of action and greater potency *in vivo* (Deacon et al., 1998). Based on the results of preclinical and clinical trials, Byetta® (Exenatide), a synthetic version of exendin-4, was approved by the U.S. Food and Drug Administration in April 2005 for adjunctive glycemic control in patients with T2DM who are taking metformin, a sulfonylurea, or a combination of metformin and a sulfonylurea (Yoo et al., 2006).

Previous studies have investigated the pharmacokinetics of exendin-4 following different administration routes in rats (Young et al., 1999; Parkes et al., 2001) and humans (Meneilly et al., 2003; Federico et al., 2005; Kolterman et al., 2005; Soon et al., 2006; Linnebjerg et al., 2007). In these preclinical and clinical trials, serum exendin-4 concentrations were mostly measured by Amylin Pharmaceuticals, Inc. (San Diego, USA) using an immunoenzymetric assay (IEMA) (Petrella et al., 2001). In this paper, we developed a radioimmunoassay (RIA) for exendin-4 with the aid of the magnetic separation system. Using this method we characterized the pharmacokinetics of exendin-4 following single and repeated s.c. administration in rhesus monkeys. To our knowledge, the pharmacokinetics of exendin-4 in nonhumans' primates has not been reported. This is the first report to evaluate the pharmacokinetics of exendin-4 in rhesus monkeys.

2. Materials and methods

2.1. Materials

The tested exendin-4 (with amino acid sequence of HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-NH_2, 4186.7 Da) was provided by Baolijian Genetic Engineering Co. Ltd. (Dongguan, China). The drug was available as white lyophilized powder and the purity was proved to be greater than 95.0% by high-performance liquid chromatography (HPLC). An artificial peptide (Tyr-exendin-4) was designed to consist of the 39-amino acid peptide extended at the C terminus so as to contain a tyrosine residue for iodination and was prepared by CL. (XIAN) Bio-Scientific Co. Ltd. (Xian, China). The purified 40-amino acid peptide was 96.5% homogeneous as determined by HPLC and the mass spectrum (MS) analyses yielded a mass of 4349.6 Da for Tyr-exendin-4 (calculated MW = 4350.8 Da). $Na^{125}I$ (99.0% purity and 638.0 GBq/mg) was purchased from Amersham Biosciences Ltd. (Amersham, UK). The other common chemicals were provided by standard commercial sources and were of the highest quality available.

2.2. Animals

Rhesus monkeys and New Zealand white rabbits were supplied by the Animal Raising Center of the Academy of Military Medical Sciences. The animals were individually housed in stainless-steel cages in a room with controlled temperature ($25 \pm 1\,^{\circ}C$) and humidity ($55 \pm 5\%$) and a 12-h light/dark cycle. The animals were fed with standard diet and had free access to water. All procedures involving animals and their care were carried out according to the guidelines of the Institutional Ethical Committee for Care and Use of Laboratory Animal of Academy of Military Medical Sciences in accordance with the governmental guidelines on animal experimentation, National Institutes of Health "Principles of Laboratory Animal Care".

2.3. Development of exendin-4 RIA

2.3.1. Immunization and preparation of antiserum

Immunisation was performed in two male New Zealand white rabbits (weighing 3.0 ± 0.2 kg). Exendin-4 (100 μg to 250 μl in physiological saline) was emulsified with an equal volume of Freund's complete adjuvant (Sigma, USA). This suspension was injected into ear vein. Booster injections of 100 μg of immunogen were given every 4 weeks as an emulsion in Freund's incomplete adjuvant (Sigma, USA). Blood samples were collected from the marginal ear vein 7–10 days after each booster injection, and the titer of the antiserum determined. The rabbits were bled out under deep anaesthesia when the antibody titer reached a satisfactory level.

Purifications of antiserum on 1 ml protein A column (Amersham, UK) were carried out as recommended by the manufacturer's protocols. Antiserum from rabbits were diluted with tris(hydroxymethyl)aminomethane buffer (Tris buffer, pH 8.0) and loaded onto the protein A column. The column was subsequently washed with Tris buffer (pH 8.0) until OD_{280} returned to baseline and retained antibodies were eluted with 5 ml glycin buffer (50 mmol/l, pH 3.0). The eluted antibodies were immediately neutralized with Tris buffer (pH 8.0), rapidly frozen and stored at $-20\,^{\circ}C$ prior to use.

2.3.2. Radioiodination of Tyr-exendin-4 and purification

A tracer was produced by iodination of Tyr-exendin-4 with ^{125}I, using the chloramine T method (Hunter and Greenwood, 1964). Briefly, 18.5 MBq of $Na^{125}I$ in 10 μl of 0.1 mol/l sodium hydroxide was added to a 1.5 ml plastic tube containing 2 μg of Tyr-exendin-4 in 50 μl PBS (pH 7.4) on ice. Then 25 μg of chloramine T (10 μl) solution in PBS (pH 7.4) was added. After 5 s of mixing, the mixture was incubated for another 25 s at room temperature. Iodination of the peptide was stopped by addition of 100 μl acetic acid buffer (0.1 mol/l, pH 4.0). The final mixture was loaded onto a 1 cm × 29 cm Sephadex G-15 column (Amersham, UK) for the separation of unincorporated free iodide from ^{125}I-labeled Tyr-exendin-4. The radioiodinated materials were eluted with acetic acid buffer (0.1 mol/l, pH 4.0),

at a flow rate of 1 ml/min. Fractions of 1 ml were collected into polystyrene tubes and a 5 μl aliquot was removed from each fraction and surveyed for radioactivity in a Radiomatic™ Flow Scintillation Analyzer (Packard 525TR, USA). The fractions containing ^{125}I-Tyr-exendin-4 were pooled and used as tracer. Specific radioactivity was 8.3 MBq/μg (223.4 μCi/μg).

2.3.3. Antibody screening

Assays to screen and titer antibodies were performed as follows in order to determine appropriate working dilutions. Various concentrations of the antiserum in assay buffer (as described below) were incubated with ^{125}I-Tyr-exendin-4 (25,000 cpm in 100 μl assay buffer). After equilibration at 4 °C for 48 h, the separation of bound and free ligand was achieved as described in the RIA protocol below and the proportion of added ^{125}I-Tyr-exendin-4 that was bound to the antiserum was calculated. A final dilution of 1:30,000 was chosen for the rabbit antiserum.

2.3.4. Exendin-4 extraction

A solid-phase extraction was employed prior to RIA. Sep-Pak C_{18} cartridges (Waters Associates, USA) were equilibrated by washing with 1 ml of 60% acetonitrile in 1% trifluoroacetic acid (TFA) followed by 3 ml of 1% TFA three times. The serum samples from monkeys were acidified with a double volume of 1% TFA, mixed, and centrifuged at 3000 × *g* for 20 min at 4 °C. The supernatant was loaded onto the cartridge and was eluted with 4 ml of 80% isopropanol in 0.1% TFA, and collected into polystyrene tubes. The eluates were dried in a centrifugal concentrator and the dry extract reconstituted in assay buffer for RIA.

2.3.5. RIA procedure

Calibration standards were prepared over the range 25–2000 pg/ml exendin-4 in pooled monkey serum. A zero serum calibration standard and serum quality control (QC) samples at three levels (50, 200 and 1000 pg/ml) were also analyzed. All serum samples were extracted, as described above, before RIA. Analysis of the extracts was carried out in triplicate using polypropylene tubes (12 mm × 75 mm) for all calibration standards, quality controls and test samples in which the concentration of exendin-4 was to be determined.

Disequilibrium conditions were chosen for the assay and the magnetic separation method was used for the separation of free and antibody bound ^{125}I-Tyr-exendin-4. The assay buffer used was 0.05 mol/l PBS (pH 7.5) containing 0.05 mol/l EDTA, 0.05% sodium azide (w/v) and 1.0% BSA (w/v). To set up an assay, 100 μl of exendin-4 antiserum (1:30,000 dilution) was added to the tubes, followed by 100 μl calibration standards, quality controls or test samples, 200 μl RIA assay buffer. After a 48 h incubation at 4 °C, ^{125}I-Tyr-exendin-4 (25,000 cpm) in 100 μl assay buffer was added and the incubation was continued for another 48 h at 4 °C. The separation of bound and free ligand was achieved by the addition of 100 μl goat anti-rabbit IgG magnetic beads (New England Biolabs Inc., USA) while mixing in an ice water bath. The mixtures were incubated with agitation at 4 °C for 2 h and then placed in a magnetic separation rack (New England Biolabs Inc., USA). The magnet aggregated the beads to one side of the tube such that the supernatants were easily decanted and the antibody-bound radioactivity in the magnetic beads was counted by scintillation counter as the bound radioactivity (*B*). Fig. 1 illustrated the steps of solid-phase radioimmunoassay with magnetic separation. The total radioactivity (*T*) was determined by radioactivity measurement of 100 μl of ^{125}I -Tyr-exendin-4 in assay buffer. Non-specific binding (NSB) was determined by substituting the antiserum with an equal amount of assay buffer. Tubes without exendin-4 were used to assess the maximum binding (B_0) of the tracer to the antibody. The results were expressed as the ratio (%) between the amounts of tracer bound to antibody in the presence (*B*) and in the absence (B_0) of unlabeled exendin-4. Using four-parameter logistic curve fit regression, a calibration curve of control binding [(B/B_0)%] versus logarithmic concentration of exendin-4 was constructed. The concentration of exendin-4 in test samples was calculated by interpolation from the calibration curve.

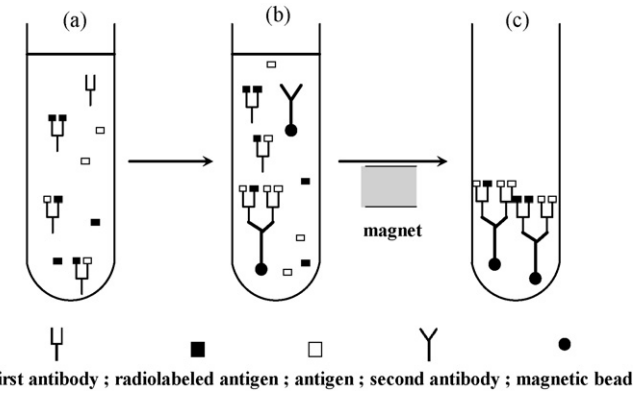

Fig. 1. Radioimmunoassay with magnetic separation protocol. (a) Radiolabeled antigen and unlabeled antigen competed for the binding sites of the first antibodies; (b) add the second antibody coupled to magnetic bead; (c) apply magnetic separation rack and the antibody-bound radioactivity in the magnetic beads is counted.

2.3.6. RIA validation

The RIA was validated in accordance with guidelines (Dadgar et al., 1995; Shah et al., 2000) that establish the performance of an assay with regard to its specificity, sensitivity, accuracy, precision, etc.

We evaluated the specificity of the RIA by determining the relative potency of the following peptides: glucagon-like peptide (GLP), erythropoietin (EPO), parathyroid hormone(1–34) PTH(1–34) and brain natriuretic peptide (BNP). The relative potency was calculated by dividing the quantity of cross-reactants displacing 50% of the antibody-bound ^{125}I-Tyr-exendin-4 by the quantity of exendin-4 producing 50% of displacement. These peptides obtained from Baolijian Genetic Engineering Co. Ltd. (Dongguan, China) and the purities were proved to be greater than 95.0% by HPLC.

Sensitivity was defined as the minimum amount of unlabeled exendin-4 that caused a reduction in the percent of tracer bound to the antibody greater than twice the standard deviation (S.D.) of 10 determinations of B_0.

Intra-day assay accuracy and precision were calculated from a single run of 10 replicates of QC samples with concentrations of 50, 200 and 1000 pg/ml exendin-4 in serum. Inter-day assay performance was calculated from six separate runs of five replicates with concentration of 50, 200 and 1000 pg/ml exendin-4 in serum.

To study the effect of dilution on the calibration range, serum samples with high concentration were prepared and diluted (10-, 25- and 100-fold) with assay buffer before assay. Accuracy was determined by comparing obtained with theoretical values.

Serum samples at concentrations of 50, 200 and 1000 pg/ml were investigated for stability under three sets of conditions relevant to pharmacokinetics research, namely storage periods prior to analysis of 1 day at room temperature, 1 week at 4 °C and 1 month at −20 °C.

2.4. Rhesus monkeys study

2.4.1. Experimental design and dosage groups

Nine male rhesus monkeys (weighing 4.3 ± 0.7 kg) were used in pharmacokinetics experiments. In single-dose studies, the monkeys were randomly divided into three groups based on their body weights. Animals were administered a single s.c. injection at either 1, 3, or 10 μg/kg. The 10 μg/kg dose group was designed for multiple s.c. doses (10 μg/kg, once daily for 7 consecutive days), and the 3 μg/kg dose group was cross-overly designed for comparing the pharmacokinetics following i.v. administration (3 μg/kg). There was a 7-day wash-out time between two separated administration periods. Dose volumes were calculated based on the pretreatment body weights of animals, rounded to the nearest 0.01 ml and measured carefully in calibrated syringe to minimize the variability of dosing.

For monkeys received an i.v. administration, the blood samples were drawn from the femoral veins of the animals using a puncture needle at 2, 5, 10, 15, 30, 45 and 60 min and 1.5, 2, 2.5, 3, 4, 6 and 8 h after dosing. For s.c. administration groups, blood samples were drawn from the femoral veins of the animals using a puncture needle at 5, 10, 15, 30, 45 and 60 min and 1.5, 2, 3, 4, 5, 6, 8 and 12 h after dosing from all groups on days 1 and 7. In the multiple-dose group, samples were also collected just prior to administration and at 30 min after administration of the second to sixth dose. Freshly collected whole blood were immediately transferred to heparinized tubes, followed by centrifugation (1800 × *g* for 15 min at 4 °C) to separate serum. Serum samples were collected and kept at −20 °C before analysis.

2.4.2. Pharmacokinetic analysis

Pharmacokinetic data analysis was performed by the non-compartmental method. The maximum serum concentration (C_{max}) and the time to C_{max} (T_{max}) were determined from the observed serum concentrations of exendin-4. The terminal elimination half-life ($t_{1/2}$) was calculated as $0.693/k_{el}$, where the k_{el} was apparent elimination rate constant of exendin-4 from serum. The area under the serum concentration–time curve ($AUC_{0-\infty}$) from zero to infinity was calculated as the sum of $AUC_{0-t} + AUC_{t-\infty}$. AUC_{0-t} from zero to the last measurable time was calculated by trapezoidal rule and $AUC_{t-\infty}$ was calculated as C_t/λ_n, where C_t was the last observed serum concentration after administration and λ_n was elimination constant calculated from the slope of the terminal phase of the serum concentration curves. The apparent total clearance (CL) was calculated as dose/$AUC_{0-\infty}$. The volume of distribution at steady state (V_{ss}) and the mean residence time (MRT) were calculated by the non-compartmental method. The extent of absolute bioavailability (F) was calculated as (mean $AUC_{0-\infty}$ s.c./mean $AUC_{0-\infty}$ i.v.) × (dose i.v./dose s.c.) × 100%. In the multiple-dose study, all pharmacokinetic parameters were evaluated for both on days 1 and 7. The accumulation ratio (AR) was calculated as the ratio of pharmacokinetic parameters on days 7 to 1 values using C_{max} ($AR_{C_{max}}$) and AUC_{0-12} (AR_{AUC}).

2.4.3. Statistical analysis

All parameters are expressed as mean ± S.D. unless noted. Dose proportionality after a single s.c. administration of different dosages was evaluated by comparison of the dose-normalized C_{max} and $AUC_{0-\infty}$ across dosage levels using an ANOVA and linear regression analysis. Statistical analyses was performed using Origin 6.0 (Microcal Software Inc., USA). A P-value below 0.05 indicates significant difference between data means.

3. Results

3.1. Radiolabeled Tyr-exendin-4 and antiserum

Tyr-exendin-4 was synthesized with an additional tyrosine to permit ^{125}I-labeling. The iodination of Tyr-exendin-4 by the chloramine T method produced a ^{125}I-radiolabeled Tyr-exendin-4 tracer with a specific radioactivity of 8.3 MBq/μg (223.4 μCi/μg). Incorporation of >85% of ^{125}I into Tyr-exendin-4 was obtained, which eluted as a significant peak (Fig. 2). Labeled peptide bound to excess antibody approached 95% indicating that the addition of ^{125}I to Tyr-exendin-4 did not

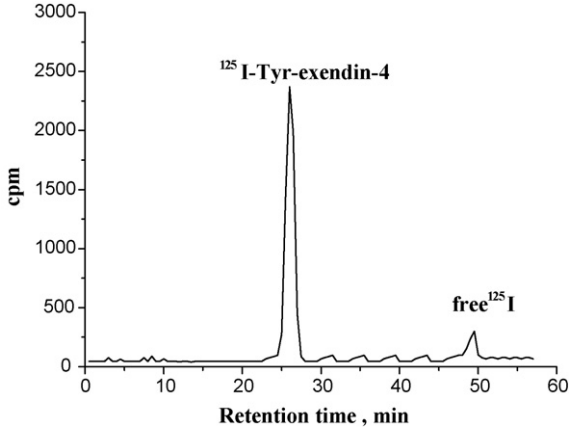

Fig. 2. Radiochromatogram profile of iodinated Tyr-exendin-4 on Sephadex G-15 column. In the radiochromatogram profile, two distinct peaks occurred with respective retention time. The first one represented the activity of the ^{125}I-Tyr-exendin-4 (25.8 min), whereas, the second peak (48 min) showed activity of the unincorporated iodine (free ^{125}I).

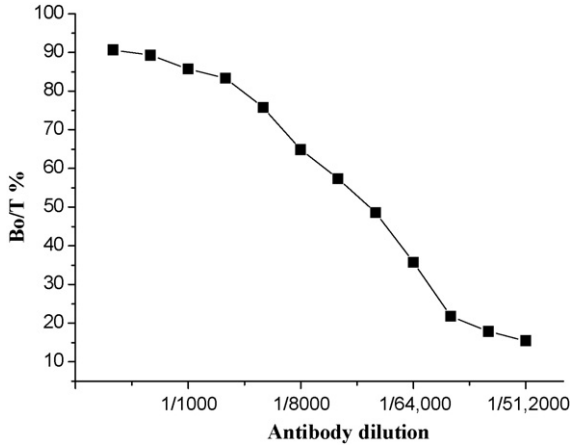

Fig. 3. Titration curves for exendin-4 to rabbit antiserum. Various concentrations of antiserum were used vs. a constant amount of ^{125}I-Tyr-exendin-4 (25,000 cpm). Each point of the curve represented the mean ± S.D. of triplicate measurements.

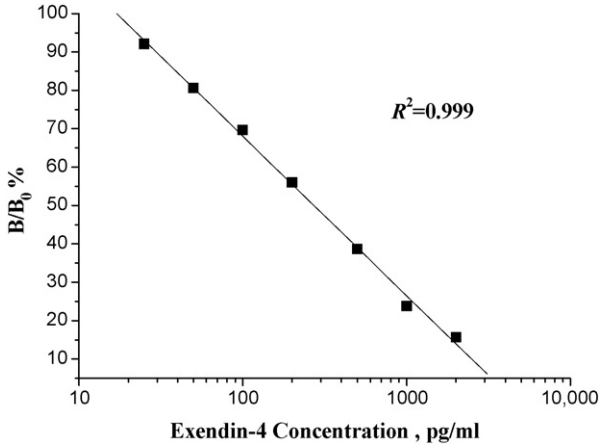

Fig. 4. Typical standard curve for exendin-4. The standard curve was fitted using a four-parameter logistic algorithm (R^2 = 0.999). The concentration range of the standard curve was between 25 and 2000 pg/ml. Each point of the curves represented the mean ± S.D. of triplicate measurements.

reduce its immunoreactivity. The tracer allowed a highly sensitive exendin-4 RIA with low NSB (<2.0%) and high-specific binding.

Anti-exendin-4 antiserum with similar affinity and specificity for exendin-4 were obtained from two rabbits. The antiserum issued from the screening demonstrated a high affinity for ^{125}I-Tyr-exendin-4 with an apparent affinity constant K_a equal to 3.2×10^{10} l/mol calculated by the method of Adrion. Titration of the antiserum showed 50% binding (B_0/T) at a dilution of 1:25,600 for the assay in monkey serum, but this was altered to 1:30,000 for the assay in the assay buffer to obtain the same sensitivity (Fig. 3).

3.2. RIA procedure and validation

A typical standard curve for ^{125}I-Tyr-exendin-4 binding to exendin-4 antiserum by adding exendin-4 (25–2000 pg/ml) was shown in Fig. 4. The R^2 value of the standard curves was 0.999. The concentration range of the calibration standards correlated to 92.1–15.7% binding, respectively.

To show the specificity of exendin-4 RIA, increasing concentrations of GLP, EPO, PTH(1–34) and BNP were used to displace ^{125}I-Tyr-exendin-4 binding to exendin-4 antibodies, and as positive control exendin-4 was used. Fig. 5 showed that GLP, EPO, PTH(1–34) and BNP did not cross-react with exendin-4 RIA when compared to exendin-4.

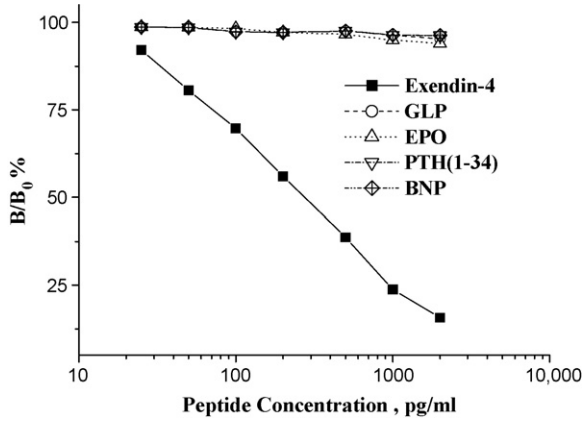

Fig. 5. Competitive displacement of ^{125}I-Tyr-exendin-4 by GLP, EPO, PTH(1–34) and BNP. Each point of the curves represented the mean of triplicate measurements.

Summary intra- and inter-assay precision and accuracy data for the determination of exendin-4 in serum were presented in Table 1. For intra-assay, the coefficient values (CV) of QC samples at concentrations of 50, 200 and 1000 pg/ml were between 6.9 and 10.3% and the accuracy of QC samples were between

Table 1
Assay precision and accuracy for the determination of exendin-4 in rhesus monkeys serum by RIA

Added serum concentration (pg/ml)	Measured concentration (pg/ml, mean ± S.D.)	Coefficient of variation (CV, %)	Accuracy (%)
Intra-assay			
1000	1014.6 ± 69.88	6.9	101.46
200	197.27 ± 13.55	7.3	98.64
50	45.78 ± 5.05	10.3	91.56
Inter-assay			
1000	1038.03 ± 74.45	7.2	103.80
200	188.35 ± 14.90	7.9	98.84
50	38.90 ± 3.92	11.3	99.58

Intra-assay was determined by analyzing multiple replicates (n = 10) spiked with exendin-4 at 50, 200 and 1000 pg/ml in the same assay. Inter-assay was determined by measuring the same spiked samples in six assays on different days.

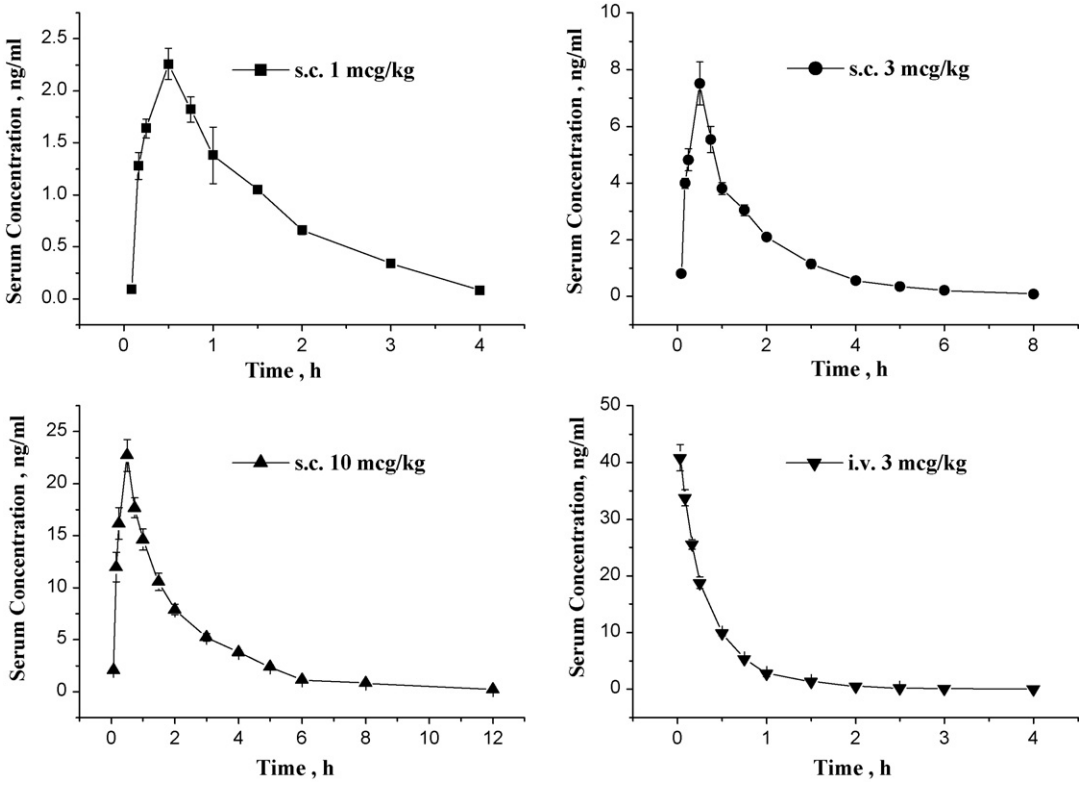

Fig. 6. The serum concentration–time profiles of exendin-4 following a single s.c. (1, 3 and 10 μg/kg, respectively) and i.v. (3 μg/kg) administration in rhesus monkeys ($n = 3$ per group). Symbols represented the observed data (mean ± S.D.).

91.56 and 101.46%. For inter-assay, the CV of QC samples were less than 11.3% and the accuracy of QC samples were between 99.58 and 103.80%.

The accuracy and precision data were used to define the limit of quantitation (LOQ) of the method. Both intra- and inter-assay precision and accuracy data for the 25 pg/ml validation sample were clearly fall within the conventional limits of acceptability for a bioanalytical method at its proposed LOQ (20% for precision and 80–120% for accuracy). Accordingly, the LOQ of the method was set at 25 pg/ml. The assay's sensitivity was determined by calculating the mean value of $10B_0$ measurements minus 2S.D. The sensitivity was 15.6 pg/ml.

Results obtained from serum samples diluted 10-, 25- and 100-fold prior to analysis showed that accuracy was from 111.2 to 98.2%, indicating the validity of determination of exendin-4 in over-range samples.

The stability of exendin-4 in serum and to a freeze-thaw cycle were studied. The recovery was 98.9–102.8 and 99.3–103.9%, respectively. The percentage difference from theoretical concentration and the difference from initial concentrations were

Table 2
Pharmacokinetic parameter following a single s.c. or i.v. administration of exendin-4 to rhesus monkeys ($n = 3$ per group)

Parameter[a,b]	Dose and route of administration			
	s.c. 1 μg/kg	s.c. 3 μg/kg	s.c. 10 μg/kg	i.v. 3 μg/kg
AUC_{0-t} (ng h/ml)	3.34 ± 0.07	11.44 ± 0.21	46.50 ± 0.00	15.20 ± 0.40
$AUC_{0-\infty}$ (ng h/ml)	3.43 ± 0.05	11.61 ± 0.22	47.10 ± 0.10	15.30 ± 0.40
MRT (h)	1.26 ± 0.01	1.67 ± 0.01	2.35 ± 0.04	0.50 ± 0.02
CL or CL/F (l/(h kg))	0.29 ± 0.01	0.26 ± 0.01	0.21 ± 0.00	0.07 ± 0.01
V_{ss} or V_{ss}/F (l/kg)	0.37 ± 0.01	0.43 ± 0.01	0.50 ± 0.01	0.03 ± 0.00
$t_{1/2}$ (h)	0.78 ± 0.05	1.33 ± 0.06	1.94 ± 0.08	0.48 ± 0.02
T_{max} (h)	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	–
C_{max} (ng/ml)	2.26 ± 0.15	7.51 ± 0.76	22.72 ± 1.54	–
F (%)	67.3 ± 5.3	75.1 ± 4.7	72.7 ± 8.4	–

[a] AUC_{0-t}: area under the concentration–time curve from zero up to last quantifiable sample; $AUC_{0-\infty}$: area under the concentration–time curve from zero up to infinity; MRT: mean residence time; CL or CL/F: total clearance for i.v. and s.c. administration route, respectively; V_{ss} or V_{ss}/F: volume of distribution for i.v. and s.c. administration route, respectively; $t_{1/2}$: half-life of elimination; C_{max}: maximum serum concentration; T_{max}: time to maximum concentration; F: absolute bioavailability.

[b] The pharmacokinetic parameters were estimated from the data shown in Fig. 6. The analyses were carried out as described in Section 2. Data were presented as mean ± S.D.

approximately equal to those of the inter-assay test. In comparison with the values for intra-assay validation, these results are acceptable for stability of the drug in monkey serum under these conditions.

3.3. Pharmacokinetics of exendin-4 in rhesus monkeys

3.3.1. Single-dose studies

Serum concentrations of exendin-4 in rhesus monkeys following s.c. dosing at 1, 3 and 10 μg/kg and i.v. dosing at 3 μg/kg were shown in Fig. 6, and corresponding mean pharmacokinetic parameters were listed in Table 2.

Following a single i.v. administration at 3 μg/kg dose, the serum concentration of exendin-4 decreased rapidly in bi-exponential manner with a terminal half-life ($t_{1/2}$) of 0.48 ± 0.02 h. The exendin-4 serum concentrations declined below the quantitation limit within 4 h after i.v. injection.

Exendin-4 reached peak serum concentration rapidly following s.c. administration with a T_{max} of 0.5 h for all three doses. Average C_{max} ranged from 2.26 ± 0.15 to 22.72 ± 1.54 ng/ml, and $AUC_{0-\infty}$ ranged from 3.43 ± 0.05 to 47.1 ± 0.10 ng h/ml at single-dose levels from 1 to 10 μg/kg. Exendin-4 elimination followed a mono-exponential profile with a $t_{1/2}$ ranging from 0.78 ± 0.05 to 1.94 ± 0.08 h. As compared to the i.v. administration at a single dose of 3 μg/kg, the absolute bioavailability after s.c. administration were estimated to be 67.3 ± 5.3, 75.1 ± 4.7 and 72.7 ± 8.4% for 1, 3 and 10 μg/kg dose, respectively.

Based on the weighted regression analysis of the pooled data, C_{max} and $AUC_{0-\infty}$ values increased as the dose increased in a linear manner (Fig. 7) but not proportional. When the dose of exendin-4 increased in a ratio of 1:3:10, the ratio of C_{max} values increased in a ratio of 1:3.3:10.1 and the ratio of $AUC_{0-\infty}$ values increased in a ratio of 1:3.4:13.7. Over the investigated dose range, increases in dosage resulted in more than proportional increases in the $AUC_{0-\infty}$, especially. This coincided with a decrease in total clearance ($P < 0.001$) and an increased elimination half-life ($P < 0.001$), consistent with non-linear disposition of the peptide. Therefore, our results support non-linear rather than linear serum pharmacokinetics of exendin-4 across the investigated dosage range in monkeys (1–10 μg/kg).

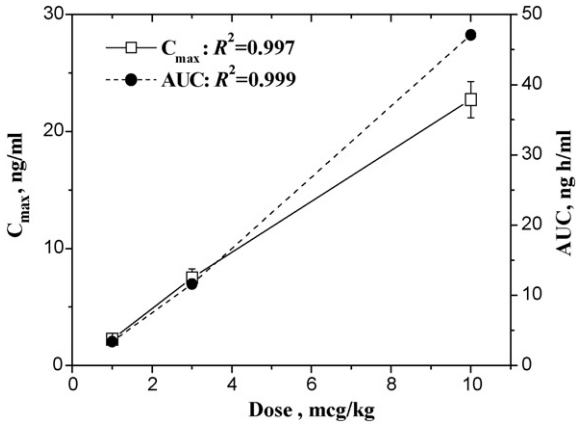

Fig. 7. The profiles of the mean serum exendin-4 C_{max} and $AUC_{0-\infty}$ after a single s.c. administration vs. the three investigated doses (1, 3 and 10 μg/kg, respectively; $n = 3$ per group) in rhesus monkeys. The R^2 for C_{max} and $AUC_{0-\infty}$ were 0.997 and 0.999.

3.3.2. Multiple-dose studies

The mean serum concentration–time profiles of exendin-4 in monkeys on days 1–7 following daily s.c. administration at 10 μg/kg for 7 consecutive days were shown in Fig. 8. The observed C_{max} and AUC_{0-12}, for the first dose on day 1 was 22.72 ± 1.54 ng/ml and 46.5 ± 0.00 ng h/ml, respec-

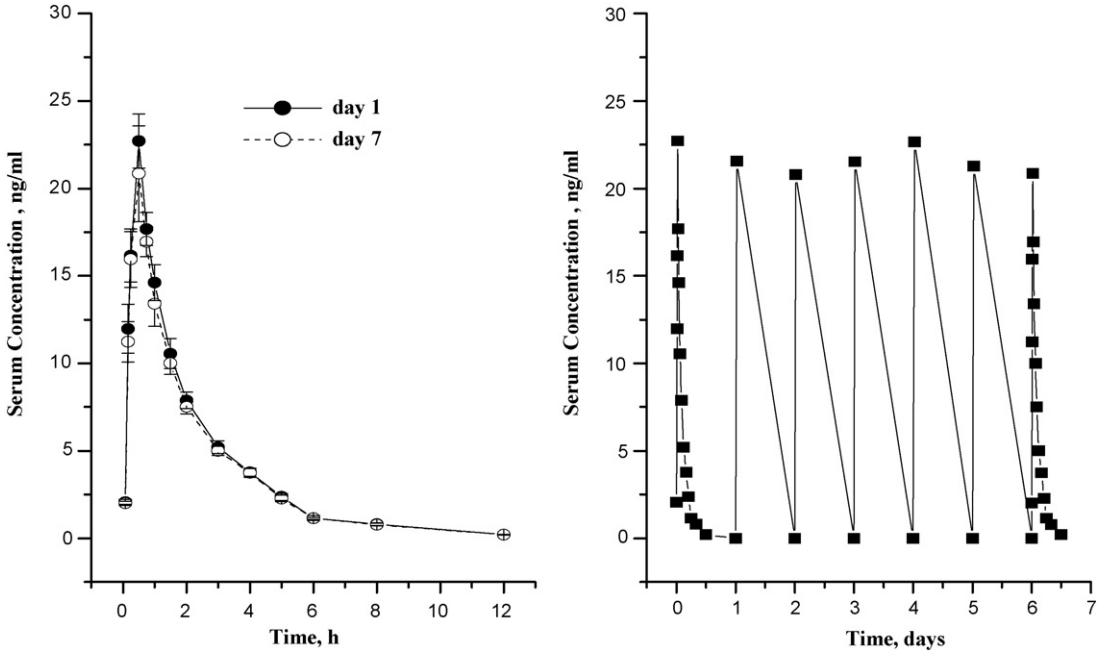

Fig. 8. The serum concentration–time profiles of exendin-4 in rhesus monkeys during and following daily s.c. administration of 10 μg/kg for 7 consecutive days. Symbols represented the observed data (mean ± S.D.).

Table 3
Day 1 vs. day 7 pharmacokinetic parameter of exendin-4 in rhesus monkeys following daily s.c. administration (10 μg/kg) for 7 consecutive days ($n = 3$)

Parameter[a,b]	Single dose (day 1)	Multiple dose (day 7)	P-values
AUC_{0-12} (ng h/ml)	46.50 ± 0.00	44.30 ± 1.30	0.1037
$AUC_{0-\infty}$ (ng h/ml)	–	44.90 ± 1.30	0.0943
MRT (h)	2.35 ± 0.04	2.38 ± 0.04	0.4437
CL/F (l/(h kg))	0.21 ± 0.00	0.07 ± 0.00	0.0980
V_{ss}/F (l/kg)	0.50 ± 0.01	0.16 ± 0.01	0.1444
$t_{1/2}$ (h)	1.94 ± 0.08	1.93 ± 0.08	0.8928
T_{max} (h)	0.50 ± 0.00	0.50 ± 0.00	1.0000
C_{max} (ng/ml)	22.72 ± 1.54	20.80 ± 2.80	0.3731
AR_{Cmax}	0.92		
AR_{AUC}	0.95		

[a] AR: accumulation ratio; other abbreviations were explained in the footnote to Table 2.
[b] The pharmacokinetic parameters were estimated from the data shown in Fig. 8. The analyses were carried out as described in Section 2. Data were presented as mean ± S.D.

tively. For the last dose on day 7, C_{max} and AUC_{0-12} were 20.80 ± 2.80 ng/ml and 44.3 ± 1.3 ng h/ml, respectively. An accumulation ratio of approximately 1.0 (with a 90% CI of 0.94–1.02) was observed based on the days 7 to 1 ratios of C_{max} and AUC_{0-12}, indicating no accumulation upon multiple doses in the monkeys. The pharmacokinetics similarities of the days 1 and 7 from multiple doses were demonstrated in Table 3 ($P > 0.05$).

4. Discussion

4.1. Exendin-4 RIA

Affinity constant values (K_a) for an antiserum normally range from 10^9 to 10^{11} l/mol (Dadgar et al., 1995). This assay produced antiserum with a K_a value of 3.2×10^{10} l/ mol, indicating sufficient binding to the radioligand. The resulting antibody dilution for the assay was set at 1:30,000. Antibody specificity was determined using GLP, EPO, PTH(1–34) and BNP. Cross-reactivities of <1% were obtained, showing good selectivity and specificity of the antibody.

Iodination is one of the most common means of radiolabeling peptides to a high-specific activity, but requires the presence of a tyrosine residue (Hunter and Greenwood, 1964; Odell et al., 1967). So Tyr-exendin-4 was designed to consist of exendin-4 extended at the C terminus so as to contain a tyrosine residue for iodination and was synthesized using solid-phase methodologies. Radioactive iodine was successfully introduced into Tyr-exendin-4 and the labeled antigen were highly purified by HPLC. The concentrated tracer (8.3 MBq/μg) was stable for at least 6 months without further purification. The immunoreactivity of ^{125}I-Tyr-exendin-4 was almost not changed, and the ^{125}I-labeled method had little effect on the immunoreactivity of ^{125}I-Tyr-exendin-4.

An essential requirement for all reliable RIA is an efficient, practical and clean method for separation of the bound and free ligand fractions (Shah et al., 2000). A significant advance in recent years has been the development of solid-phase separation method which utilize antibody immobilized on magnetic particles resulting in clean separation by the application of a magnetic field and obviate the need for centrifugation (Kemshead and Ugelstad, 1985; Bangs, 1990; Shinkai and Ito, 2004; Alexiou et al., 2006). Goat anti-rabbit IgG binds the heavy chain of all rabbit IgG subclasses and is suitable for immunoassays that employ a rabbit IgG primary polyclonal antibody. For the goat anti-rabbit IgG magnetic beads, this secondary antibody is covalently coupled to a nonporous paramagnetic particle. The diameter of the paramagnetic particles is 1 μm and the particle concentration is 3.65×10^{10} particles/ml. Magnetic beads combine high surface capacity with fast, efficient separation without the need for centrifugation, thus they provide a solid-phase methodology which avoids many of the disadvantages of centrifugation. The use of magnetic beads in this assay achieved easy separation and in some cases improved sensitivity.

The standard curve of the radioimmunoassay was reproducible and the precision was satisfactory. The high degree of parallelism found between the standard curve and the serial dilutions of extracted serum and spiked serum samples provide good evidence for the accurate and precise determination of exendin-4 levels in monkey serum. The intra- and inter-assay coefficients of variation were within the limits expected for this type of assay (Chard, 1990).

In summary, the exendin-4 radioimmunoassay showed satisfactory accuracy and precision along with good selectivity and demonstrated its usefulness as a means of quantitation of this drug in monkey serum.

4.2. Pharmacokinetics of exendin-4 in rhesus monkeys

Following a single s.c. administration at 1, 3 and 10 μg/kg, exendin-4 reached peak serum concentration rapidly with a T_{max} of 0.5 h for all three doses. The terminal $t_{1/2}$ after s.c. administration was prolonged in the monkeys (0.78 ± 0.05–1.94 ± 0.08 h) when compared with terminal $t_{1/2}$ after i.v. administration (0.48 ± 0.02 h), suggesting that absorption of exendin-4 was the rate-limiting factor in determining its pharmacokinetics after s.c. administration. As compared to the i.v. administration at a single dose of 3 μg/kg, the absolute bioavailability after s.c. administration were estimated to be 67.3 ± 5.3, 75.1 ± 4.7 and 72.7 ± 8.4% for 1, 3 and 10 μg/kg dose, respectively. The pharmacokinetic properties of exendin-4 obtained from the present study closely matched those reported in previous studies in rats (Young et al., 1999; Parkes et al., 2001) and humans (Meneilly et al., 2003; Federico et al., 2005; Kolterman et al., 2005; Soon et al., 2006; Linnebjerg et al., 2007). On the other hand, across the investigated dosage range in monkeys (1–10 μg/kg), C_{max} and $AUC_{0-\infty}$ values increased as the dose increased in a linear manner but not proportional. Our results supported non-linear rather than linear serum pharmacokinetics of exendin-4. However, the previously reported pharmacokinetic studies showed dose-proportional exendin-4 pharmacokinetics in patients with T2DM (Kolterman et al., 2005). The possible reason for this difference might due to the different sources of the tested peptide and assay methods utilized. The presence of inter-species and/or inter-strain differences might also contribute to such findings.

For multiple-dose studies, the accumulation factor was close to 1.0, indicating no accumulation upon multiple doses in the monkeys. This was similar when compared to the previous studies (Meneilly et al., 2003; Kolterman et al., 2005).

Acknowledgement

This work was supported by the National High Technology 863 project (No. 2003AA2Z347B), PR China.

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