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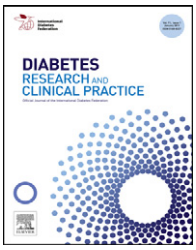

# Peptide complex containing GLP-1 exhibited long-acting properties in the treatment of type 2 diabetes

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ABSTRACT

The multiple physiological characterizations of glucagon-like peptide-1 (GLP-1) make it a promising drug candidate for the treatment of type 2 diabetes. However, *in vivo*, the half-life of GLP-1 is short, which is caused by the degradation of dipeptidyl peptidase-IV (DPP-IV) and renal clearance. Thus, the stabilization of GLP-1 is critical for its utility in drug development. Peptides known as GLP-1 protectors are predicted to increase the half-life of GLP-1 *in vivo*. Protecting peptides are able to form stable complexes by non-covalent interactions with human GLP-1. In this study, the stability of the complex was investigated, and the physiological functions of the GLP-1/peptide 1 complex were compared to those of exenatide and liraglutide in animals. The results indicated that the GLP-1/peptide 1 complex remarkably raised the half-life of GLP-1 *in vivo* and showed better glucose tolerance and higher $HbA_{1c}$ reduction than exenatide and liraglutide in rodents. Based upon these results, it is suggested that the GLP-1/peptide 1 complex might be utilized as a possible potent anti-diabetic drug in the treatment of type 2 diabetes mellitus.



## 1. Introduction

Glucagon-like peptide 1 (GLP-1) is a gut hormone released from intestinal L cells following oral glucose administration [1]. GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) contribute to approximately 60–70% of the postprandial insulin response in healthy individuals [2]. However, in the absence of GIP, GLP-1 preserves the blood glucose lowering effect [3]. Therefore, GLP-1 is vital for insulin secretion, which suggests that it could have a potential application in therapeutic strategies for type 2 diabetes treatments [4]. GLP-1 serves as an incretin factor stimulating the secretion of insulin and reduces blood glucose both in normal subjects and in subjects with type 2 diabetes mellitus [5]. GLP-1 is a potent anti-hyperglycemic hormone, stimulating the secretion of insulin in a glucose-dependent manner and suppressing glucagon secretion, which minimizes the risk of hypoglycemia [6,7]. Interestingly, when the plasma glucose concentration is in the normal fasting range, GLP-1 does not stimulate insulin, which causes hypoglycemia [4]. It was found that GLP-1

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*Abbreviations:* AUC, area under the curve; DPP-IV, dipeptidylpeptidase IV; ELISA, enzyme-linked immunosorbent assay; GLP-1, glucagon-like peptide 1; IPGTT, intraperitoneally glucose tolerance test.

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restores the glucose sensitivity of pancreatic β-cells, which possibly depend on the increasing level of glucose transporter 2 (GLUT2) and glucokinase expression [8]. It was also confirmed that GLP-1 inhibits apoptosis (programmed cell death) in cells, consequently improving their survival [9].

The deficiency of GLP-1 secretion in type 2 diabetes suggested that GLP-1 acts as a promising potential therapy [10]. Evidence has demonstrated that the infusion of GLP-1 decreases blood glucose [11], and GLP-1 has been identified as playing a crucial role in the regulation of glucose metabolism. A recent report showed that GLP-1 has an exceptionally short half-life of less than 2 min *in vivo*, which is due to rapid degradation by the enzyme dipeptidyl peptidase IV (DPP-IV) [12]. This makes the therapeutic administration of GLP-1 seem impractical; thus, many efforts have focused on amending the pharmacokinetic properties of GLP-1 in a series of derivatives and analogues. Two GLP-1 analogues, exenatide and liraglutide, were approved by the FDA for the treatment of type 2 diabetes in 2005 and 2010, respectively [13]. Exendin-4 (exenatide is a synthetic exendin-4) shares 53% amino acid sequence similarity with GLP-1, which is a 39-amino acid peptide produced in the salivary glands of the Gila monster (*Heloderma suspectum*) [14]. No specific exendin-4 receptor was detected; the effect of exendin-4 is exerted through the GLP-1 receptor [15]. Liraglutide contains two modifications: a substitution of $Arg^{34}$ for $Lys^{34}$ and an attachment of a C-16 free-fatty acid derivative via a glutamoyl spacer to $Lys^{26}$ [16]. The free-fatty acid derivative is supposed to promote the non-covalent binding of liraglutide and albumin [17]; accordingly, the absorption of liraglutide is delayed from the injection site, and clearance is also decreased [18]. In a LEAD6 study, Buse et al. compared the efficacy and safety of two GLP-1 analogues (exenatide and liraglutide) in the treatment of type 2 diabetes [19]. Liraglutide showed better glucose tolerance than exenatide and presented fewer side effects, such as nausea, than exenatide [19].

To avoid any possible immunotoxicity by non-mammalian products, it is preferable to employ human full-length GLP-1 as a tool for the treatment of type 2 diabetes in this study. Accordingly, the aim of this study is to stabilize GLP-1 *in vivo* by prolonging its half-life; an amphipathic peptide is the ideal candidate to achieve this goal. Amphipathic peptides contain the following two domains: a hydrophilic domain that interacts with negatively charged molecules and a hydrophobic domain that interacts with the cell membrane [20]. Accordingly, amphipathic peptides are employed as cell-penetrating peptides, but the uptake mechanism is still unclear and a limitation of the clinical utility of the cell-penetrating peptides. Divita et al. reported a secondary cell-penetrating peptide, CADY, which allowed the formation of a stable complex with siRNA via non-covalent interactions [21]. Nontoxic CADY-based technology has had a significant effect on the development of fundamental and therapeutic siRNA-based applications. In this research, CADY peptide acting as a protecting peptide was modified to achieve a stable complex of GLP-1 and protecting peptide. The physiological properties of the GLP-1/protecting peptide complex were investigated, including its stabilization, glucoregulatory and long-lasting anti-diabetic effects, in rodent and human serum.

## 2. Materials and methods

### 2.1. Materials

DPP IV enzyme (0.1 mg/ml; purity ~85%) was purchased from Sigma. Human GLP-1 (9-37) and goat anti-rat insulin ELISA kits were purchased from Phoenix Technology, Inc. The GLP-1 (7-37) ELISA kit was purchased from Millipore. A one-touch blood glucose meter and filters were purchased from Abbott. Other chemicals unless otherwise specified were purchased from Sigma.

### 2.2. Animals

All studies were performed with permits from the Animal Experiments Inspectorate, China. Male ZDF (fa/fa) rats, lean male ZDF rats and male Sprague Dawley (SD) rats were obtained from Shanghai Laboratory Animal Co. (SLAC), China Academy of Sciences (Shanghai, China).

### 2.3. Peptide synthesis

GLP-1 (HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR) and two protecting peptides (peptide 1: GLWWKAWWKAWWKSLWWRKRKRKA; peptide 2: GLWWKVWWKVWWKSLWWRKRLRKA) were purchased from Sangon Biotech Co. (Shanghai; HPLC-purified; purity >90%, identified by MS). The freeze-dried peptides were weighed and dissolved in pure water to make 10 mg/ml stock solutions for further analysis. Exenatide and liraglutide were kind gifts from Dr. Wei Liu in Tianjin General Hospital.

### 2.4. Preparation of blood samples

Human whole blood from healthy individuals and rat blood samples were directly drawn into P800*, $K_2$EDTA tubes (BD, Franklin Lakes, NJ) by venipuncture. Blood sample was immediate centrifugation for 20 min at 11,903 × *g* for obtaining serum. The serum samples were stored at −80 °C for further use.

### 2.5. Analysis of the mixture of GLP-1 and protecting peptides by HPLC

GLP-1 and protecting peptides (peptide 1 or peptide 2) were mixed at various ratios and then incubated at 25 °C for 5 min. Different amounts of peptides were applied: 100 μg/ml, 500 μg/ml, 1 mg/ml and 2 mg/ml, while the concentration of GLP-1 remained at 100 μg/ml. The final ratios of GLP-1 to peptide were 1:1; 1:5; 1:10 and 1:20, respectively. Mixtures (10 μl) were analyzed by a Surveyor HPLC system through a C18 analytical column. The column was eluted at a flow rate of 0.5 ml/min in a gradient mode with the mixture of mobile phase A ($H_2O$ + 40% acetonitrile + 0.1% trifluoroacetic acid) and mobile phase B (acetonitrile + 0.1% trifluoroacetic acid). Mobile phase A was eluted for 10 min, and thereafter, mobile phase B was increased from 40% to 100% in a 60-min period. HPLC analyses were performed at ambient temperature and the UV detection wavelength was set at 220 nm. Ten microliter

aliquots of GLP-1 stock solution and protecting peptide stock solution were injected into the C18 column of HPLC as the controls, respectively.

### 2.6. Electrospray ionization mass spectrometry

Pneumatically assisted ESI mass analysis was performed in a Perkin-Elmer Sciex API III Plus triple quadrupole mass spectrometer system. The potential of the electrospray needle was placed at 3000 V to produce positive ions, and the potential of the orifice leading into the mass analyzer was set at 50 V extract positive. The flow rates for the nebulizer gas (oxygen) and the curtain gas (nitrogen) were both set at 350 l/h for positive ions. The instrument was mass-calibrated in the positive ionization mode with a mixture of polypropylene glycols. The samples were analyzed in an aqueous solution of 50% $H_2O$/50% ACN.

### 2.7. Stabilization analysis of GLP-1/peptide complexes

The GLP-1/peptide complexes were prepared at the proportions of 1:10 for peptide 1 or peptide 2 and then incubated with human serum (final sample volume of 1 ml; final GLP-1 mass of 750 μg) for 5 min to 250 h. Fifty-microliter aliquots from the incubated mixtures were analyzed by anti-human (9–37) ELISA kits (R&D Diagnostics) to quantitatively determine the half-life of GLP-1 in human serum following the manufacturer's instruction. The *in vivo* half-lives of GLP-1/peptide complexes were identified in Sprague Dawley rats. Sprague Dawley rats ($n = 5$ per group, male) were subcutaneously injected with GLP-1/peptide 1 (1:10, containing of 750 μg GLP-1/kg body weight) and GLP-1/peptide 2 (1:10, containing of 750 μg GLP-1/kg body weight), respectively. Saline was subcutaneously injected into a control group ($n = 3$). Blood samples (200 μl) were subsequently obtained from treated and control groups within the experimental period of 7 days. The obtained serum samples were analyzed using the GLP-1 (7-37) ELISA assay (R&D Diagnostics) to confirm the half-life of GLP-1 in rats.

### 2.8. Insulin secretion assay

Sprague Dawley rats were subjected to an insulin secretion measurement after an overnight fast ($n = 6$ per group). Two complexes of GLP-1/peptide 1 and GLP-1/peptide 2 were subcutaneously injected into the rats so that each animal received 100 μg f GLP-1. A certain amount of GLP-1 (100 μg) and saline were subcutaneously injected as controls. Glucose (10 g/kg body weight) was orally administered after 30 min. Blood samples were collected by tail vein incision at 2, 5, 10, 15, 30, 45, 60, 90, 120, 150 and 180 min from the beginning of glucose administration. This procedure allowed for collection without cutting the skeletal component of the tail and did not require the catheterization of a blood vessel. Blood samples were then assayed for insulin levels using the insulin ELISA kit.

### 2.9. Glucoregulatory effect of GLP-1/peptide complexes

To determine if the GLP-1/peptide complex has any glucoregulatory effects *in vivo*, Sprague Dawley rats ($n = 5$ per group) were subcutaneously injected with GLP-1/peptide 1 (1:10, containing of 100 μg GLP-1) or GLP-1/peptide 2 (1:10, containing of 100 μg GLP-1). Saline was injected into the control group. Glucose (2 g/kg) was intraperitoneally injected after 10 min. Glucose was measured 30 min after glucose administration using a One-Touch Glucose Meter.

### 2.10. Glucose tolerance test upon single dose injection

Fasting Sprague Dawley rats ($n = 6$ per group) were given a single dose of GLP-1/peptide complexes (GLP-1 ~300 μg/kg body weight) intraperitoneally 30 min prior to glucose administration. Saline was injected into controls. The rats were given 2 g glucose/kg body weight via intraperitoneal injection. Blood was drawn from the tail vein, and glucose levels were measured using a glucometer at 30 min after glucose administration. Glucose (2 g/kg body weight) was administrated 30 min prior to each blood glucose measurement time point in a 96-h experimental period.

### 2.11. Glucose tolerance test upon multiple-dose injection and $HbA_{1c}$ measurement

Male Zucker Diabetic Fatty (ZDF) rats ($n = 6$ per group) were treated with GLP-1/peptide 1 complex (GLP-1 ~300 μg/kg/4 days), exenatide (100 μg/kg/12 h) and liraglutide (300 μg/kg/daily) for 28 days. The control group (lean male ZDF rats) was injected with saline. At the end of 28 days, drug treatment was ceased and blood samples were collected 2 days afterward. $HbA_{1c}$ was assessed using the DCA 2000 analyzer (Bayer Diagnostics).

### 2.12. Statistical analysis

Student's *t*-test was employed for the analyses of the data. Unless otherwise stated, the results were reported as the means ± standard error. *P* values less than 0.05 were considered significant.

## 3. Results

### 3.1. Identification of GLP-1/peptide complexes

In this study, the analysis of GLP-1/peptide complex formation was performed by HPLC. Fig. 1A showed that purified GLP-1 had a retention time of 6.7 min, and purified peptide 1 possessed a retention time of 3.2 min (Fig. 1B). When GLP-1 and peptide 1 were mixed at a ratio of 1:1, two peaks remained at 3.2 min and 6.7 min; no extra peak was detected in the spectrum (Fig. 1C). At a ratio of 1:5, the peaks of GLP-1 and peptide 1 almost disappeared, and a peak was visible with a retention time of 18.8 min (Fig. 1D). The peak of GLP-1 completely disappeared at the ratios of 1:10 and 1:20, suggesting that all GLP-1 interacted with peptide 1, as shown in Fig. 1E and F. The HPLC results predicted that the visible peak with a retention time of 18.8 min might be the GLP-1/peptide 1 complex. The HPLC spectra of the mixture of GLP-1 and peptide 2 were similar to those of the GLP-1 and peptide 1 mixture (Supplement Fig. 1).

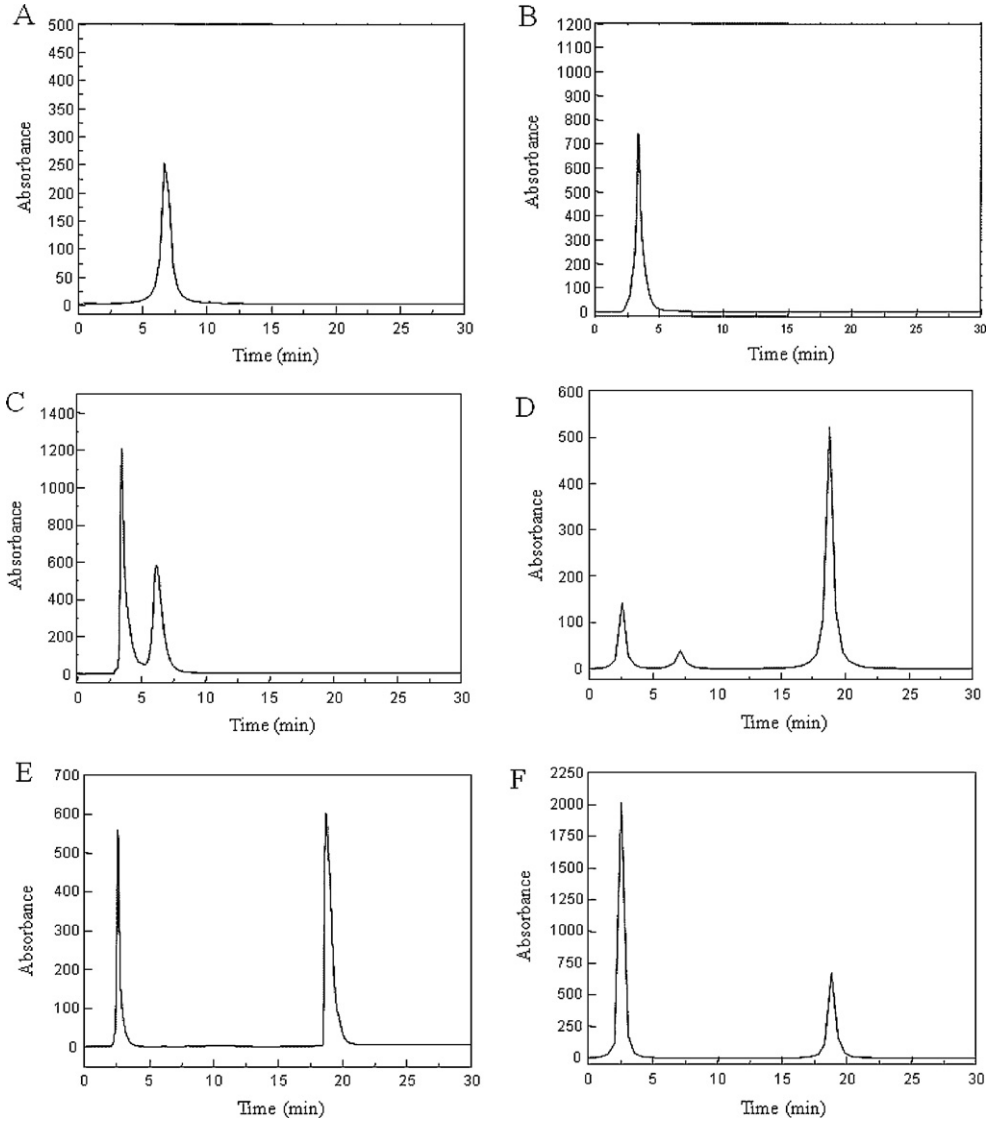


**Fig. 1 – HPLC comparison of GLP-1, protecting peptide 1 and GLP-1/protecting peptide 1 mixed by different ratios. Panel A. HPLC spectrum of GLP-1. Panel B. HPLC spectrum of protecting peptide 1. Panel C. HPLC spectrum of the mixture of GLP-1 and peptide 1 at the ratio of 1:1. Panel D. HPLC spectrum of the mixture of GLP-1 and peptide 1at the ratio of 1:5. Panel E. HPLC spectrum of the mixture of GLP-1 and peptide 1at the ratio of 1:10. Panel F. HPLC spectrum of the mixture of GLP-1 and peptide 1 at the ratio of 1:20. Conditions: samples were injected into C18 column of HPLC at 0.5 ml/min in a gradient mode by using the mixture of mobile phase A ($H_2O$ + 40% acetonitrile + 0.1% trifluoroacetic acid) and mobile phase B (acetonitrile + 0.1% trifluoroacetic acid). UV detector was set at 220 nm. Legend: the retention time of purified GLP-1 and peptide 1 were 6.7 min (A) and 3.2 min (B). A peak, which had a retention time of 18.8 min, was visible upon the increasing ratio of GLP-1 and peptide 1 (C and 1D). At 1:10 and 1:20, the complete disappearance of GLP-1 and appearance of a peak at 18.8 min indicated GLP-1 might interact with protecting peptide and formed a stable complex (E and 1F).**

To investigate the visible peak with a retention time of 18.8 min, it was disconnected by ammonium acetate and analyzed by HPLC. The results in Fig. 2A showed that no peak at 18.8 min was detected, but two peaks appeared with the retention time of 3.2 min and 6.7 min, which corresponded to the retention time of peptide 1 and GLP-1, respectively. The elution was further analyzed by mass spectrometry and subsequent SDS–PAGE. Mass spectrometry results indicated that the mass of the earlier peak was 3329.10 (full-length peptide 1), and the later peak had a mass of 3353.80 (full-length GLP-1), as shown in Fig. 2B. Two peptide bands were detected by SDS–PAGE, which are in accordance with the molecular weight of GLP-1 and peptide 1. These confirmed that the peak at 18.8 min is the complex of GLP-1 and peptide 1.

To identify the optimal mixing proportion of GLP-1 and peptide 1/peptide 2, their extinction coefficient constants were determined at 220 nm. The extinction coefficient constants of GLP-1, peptide 1 and peptide 2 were 8540 $cm^{-1} M^{-1}$, 38,750 $cm^{-1} M^{-1}$ and 40,020 $cm^{-1} M^{-1}$ at 220 nm, respectively (determined by previously described methods) [22]. In

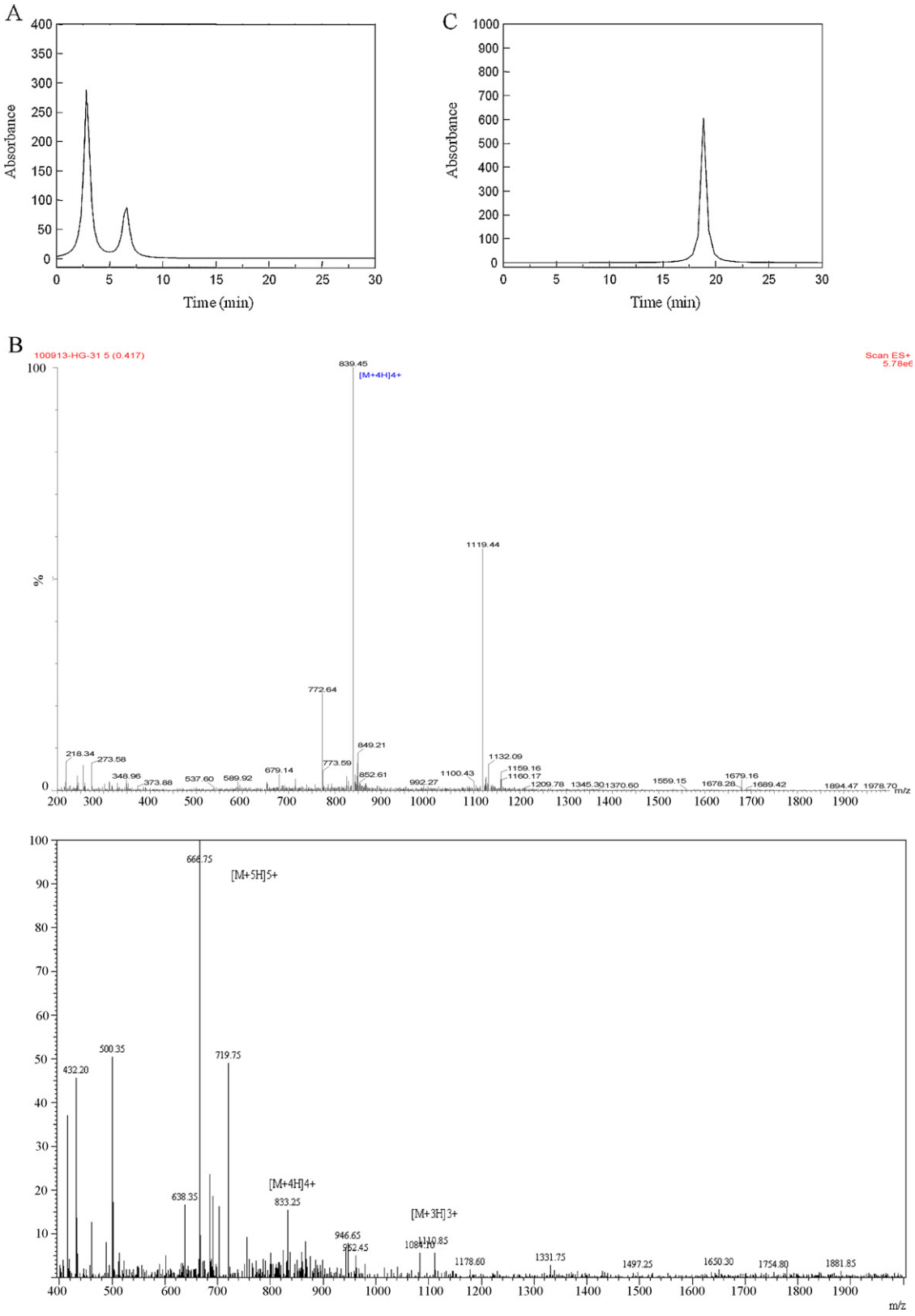


**Fig. 2 – Identification of GLP-1/peptide 1 complex and their optimal mixing ratio by HPLC. Panel A. HPLC spectrum of dispatched GLP-1/peptide 1 complex. Conditions: the presumed GLP-1/peptide 1 complex which had a retention time of 18.8 min in HPLC spectrum was collected. The obtained sample was incubated with ammonium acetate in order to unfold the complex, thereafter was loaded into C18 column of HPLC. Legend: the peak at 18.8 min disappeared, instead of the appearance of two peaks at 3.2 min and 6.7 min. They are corresponding with the retention time of peptide 1 monomer and GLP-1 monomer, respectively. Panel B. Mass spectrometry chromatograms of disconnected GLP-1/peptide 1 complex.**

combination with Fig. 2A, the optimal ratio of GLP-1 and peptide 1 was presumed as 1:6.5. Further identification was performed by HPLC analysis at a ratio of 1:6.5. As shown in Fig. 2C, the peaks of GLP-1 and peptide 1 were invisible, whereas the peak of the complex possessed the retention time of 18.8 min. The same result was obtained by application of a mixing ratio of 1:7.0 (GLP-1: peptide 2). HPLC results demonstrated that either peptide 1 or peptide 2 were able to form a complex with GLP-1. In addition, GLP-1/peptide 1 and GLP-1/peptide 2 complexes possessed the same retention times of 18.8 min in HPLC spectra.

### 3.2. Stabilization studies of the GLP-1/peptide complexes

To investigate whether the complexes of GLP-1/peptide are more resistant to serum DPPIV than GLP-1, stability assay was conducted using the GLP-1 (9–37) ELISA kit. As shown in Fig. 3A, the half-life of GLP-1 inside the GLP-1/peptide 1 complex was 90 h and inside the GLP-1/peptide 2 complex was 75 h in human serum. However, the half-life of the GLP-1 monomer was only 2.5 min. This implied that the GLP-1/peptide complexes displayed much slower decay rates compared to the GLP-1 monomer. In other words, GLP-1/peptide complexes are relatively resistant to the degrading enzyme(s) in human serum.

An *in vivo* assay was also performed to determine the stabilization and circulating concentration of GLP-1 using the GLP-1 (7–37) ELISA kit. Data in Fig. 3B demonstrated that after a single dose administration in Sprague Dawley rats, there was a significant increase in the concentration of GLP-1. The concentration of GLP-1 reached a plateau at 10 h and gradually decreased but remained detectable until 150 h. It was also shown that the GLP-1/peptide 1 and GLP-1/peptide 2 complexes have similar half-lives in human serum but have different circulating amounts in animals. The calculated $AUC_{GLP\text{-}1}$ from Fig. 3B indicated that the amount of GLP-1 inside GLP-1/peptide 1 was twice as much as that inside GLP-1/peptide 2 (Fig. 3C). It is obvious that both protecting peptide 1 and 2 are able to extend the half-life of GLP-1 in human serum and rats. In addition, the stabilization of the GLP-1/peptide 1 complex is higher than that of GLP-1/peptide 2 complex in rats.

### 3.3. Effects of GLP-1/peptide complexes on insulin secretion and glucoregulation

The insulin secretion level and glucoregulatory effects of the GLP-1/peptide complexes were examined to investigate whether they still retained the biological activities of the GLP-1 monomer. Fig. 4A showed that the oral administration of glucose (10 g/kg body weight) increased insulin levels in the rats treated with GLP-1/peptide complexes. The secreted insulin level was dramatically raised to $956.7 \pm 37.84$ pmol/l (GLP-1/peptide 1) and $827.9 \pm 31.40$ pmol/l (GLP-1/peptide 2). The levels of secreted insulin, which were induced by the GLP-1/peptide 1 complex or the GLP-1/peptide 2 complex, peaked at 60 min and returned to baseline at 180 min. Additionally, in rats treated with GLP-1 monomer, insulin levels increased to $854.12 \pm 39.82$ pmol/l within 20 min. In comparison, the rats treated with GLP-1 showed a shortened insulin secretory response, returning to baseline 90 min after glucose infusion. The increased insulin level together with the prolonged secretory response of insulin secretion observed in the Sprague Dawley rats resulted in much greater insulin levels over the course of the experiment (180 min). The $AUC_{insulin}$ calculated from the injection of glucose to the end of the test (180 min) showed that Sprague Dawley rats injected with GLP-1/peptide complexes had a 2-fold increase in insulin levels, when compared to rats injected with GLP-1, $P < 0.01$ (Fig. 4B).

The glucoregulatory effects of GLP-1/peptide complexes *in vivo* were investigated in Sprague Dawley rats. Fig. 5A showed that in saline treated rats, the concentration of blood glucose rapidly peaked at $11.34 \pm 0.55$ mmol/l after 1 min and was maintained at a high level for 30 min. Nevertheless, the rats injected with GLP-1/peptide complexes exhibited efficient glucose regulation. Blood glucose reached the maximum level 1 min after the administration of glucose and then declined to $6.66 \pm 0.51$ mmol/l (GLP-1/peptide 1) and $6.55 \pm 0.61$ mmol/l (GLP-1/peptide 2) after 10 min, $P < 0.01$. The level of glucose in the two animal groups injected with different complexes varied during the first 5 min of decline. As expected, within 5 min, the GLP-1/peptide 1 complex had decreased glucose levels at a faster rate than the GLP-1/peptide 2 complex did. Compared to GLP-1 treated rats, there were 1.6-fold increases in the $AUC_{glucose}$ value of GLP-1/peptide complex treated rats (GLP-1/peptide and GLP-1/peptide 2, Fig. 5B). These results demonstrated that GLP-1 inside of a protecting peptide preserves the insulin secretion response and glucose regulatory effects of GLP-1.

### 3.4. Long-lasting effect of GLP-1/peptide complex on glucose tolerance

To examine whether GLP-1/peptide complexes have long-lasting effects on glucose tolerance, intraperitoneal glucose tolerance tests (IPGTT) were performed for 96 h after a single dose administration of GLP-1/peptide 1, GLP-1/peptide 2, exenatide and liraglutide in Sprague Dawley rats ($n = 6$ per

**Conditions: samples were analyzed by ESI-QTOF-MS system, positive ions were produced by electrospray needle at 3000 V in an aqueous solution of 50% $H_2O$/50% ACN. Legend: average molecular mass of 3355.74 Da in top tracer was detected by the formation of the quadruple ($[M+4H]^{4+}$, *m/z* 839.45), and triple-charged molecular ions ($[M+3H]^{3+}$, *m/z* 1119.44). The result indicated that the earlier peak, which had a retention time of 3.2 min, is protecting peptide 1. Average molecular mass of 3329.05 Da in bottom tracer could be detected by the formation of the ($[M+6H]^{6+}$, *m/z* 555.85), ($[M+5H]^{5+}$, *m/z* 666.85), ($[M+4H]^{4+}$, *m/z* 833.25) and($[M+3H]^{3+}$, *m/z* 1110.75), confirming that the peak with a retention time of 6.7 min is GLP-1. Panel C. HPLC spectrum of GLP-1/peptide 1 complex mixed at the ratio of 1:6.5. Conditions: GLP-1 was incubated with peptide 1 at the ratio of 1:6.5; the mixture was loaded into C18 column of HPLC afterwards. Legend: HPLC spectrum showed that the peaks of GLP-1 and peptide 1 monomers were invisible, whereas the peak of complex was clearly detectable.**

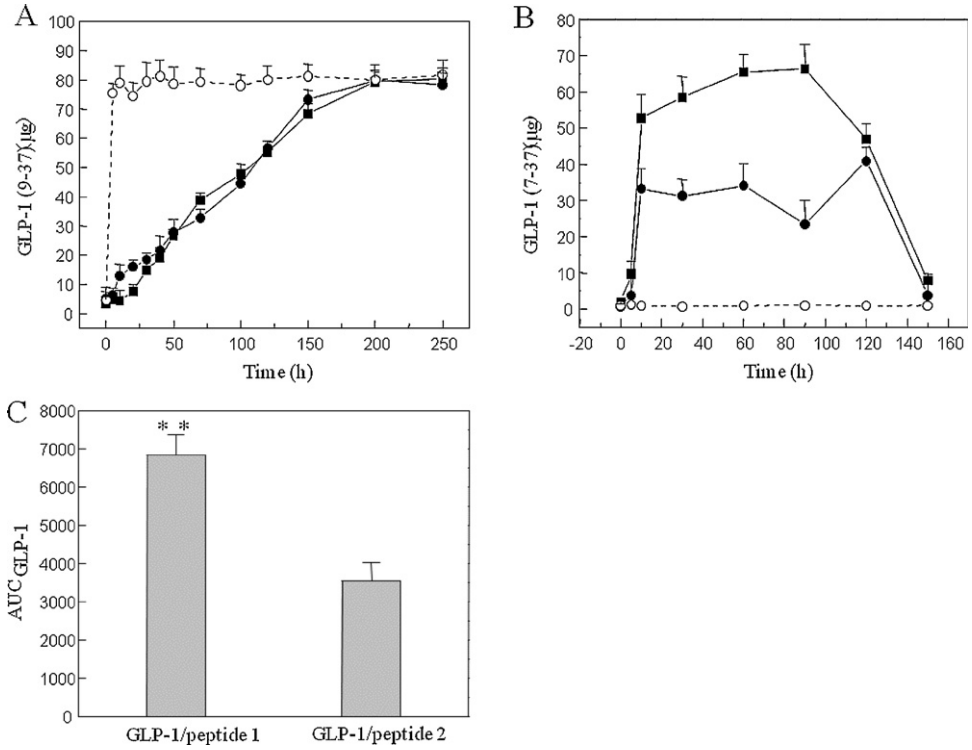


**Fig. 3 – Investigation of the half-life of GLP-1/peptide complexes *in vitro* and *in vivo*. Panel A. Half-life measurement of GLP-1 (○), GLP-1/peptide 1 (●) and GLP-1/peptide 2 complexes (■) in human serum. Conditions: the two GLP-1/peptide complexes (containing GLP-1 ~750 μg) were incubate with human serum for a 250 h experimental period. The GLP-1 (9-37) fragment was surveyed by GLP-1 (9-36) ELISA kit. Legend: result presented that the GLP-1/peptide complexes were more resistant to the degrading enzyme(s) than GLP-1 monomer. *In vitro* half lives of GLP-1 were determinate as 90 h for GLP-1/peptide 1 complex (●) and 75 h for GLP-1/peptide 2 complex (■), respectively. Panel B. Pharmacokinetic study of GLP-1 (□), GLP-1/peptide 1 complex (●) and GLP-1/peptide 2 complex (■) in SD rats. Conditions: SD rats were intraperitoneally injected with GLP-1, GLP-1/peptide 1 complex or GLP-1/peptide 2 complex (GLP-1 750 μg/kg body weight). Blood samples were taken from tail vein at interval time and the level of GLP-1 was measured by GLP-1 (7-37) ELISA kit. Legend: result indicated that GLP-1 inside of GLP-1/peptide complexes was still detectable at 120 h, whereas GLP-1 was undetectable during the whole process. Result illuminated that the two GLP-1/peptide complexes possess prolonged half-life in SD rats. Panel C. $AUC_{GLP-1}$ of the circulating concentration of GLP-1 in SD rats. Legend: the calculated $AUC_{GLP-1}$ from B found that the amount of GLP-1 inside of GLP-1/peptide 2 was half of that inside of GLP-1/peptide 1. Data are mean ± SE, $^{**}P < 0.01$.**

group). As shown in Fig. 6A, the control group showed a high blood glucose level at 12.15 ± 0.56 mmol/l in 96 h. Upon administration of exenatide, the concentration of blood glucose decreased to 6.95 ± 0.47 mmol/l at 30 min after the first glucose injection, then increased to 8.55 ± 0.36 mmol/l at 4 h. During the continued experimental period, glucose tolerance was no longer found. Both liraglutide and the GLP-1/peptide 2 complex failed to play glucoregulatory roles after 8 h. However, the rats injected with the GLP-1/peptide 1 complex had better glucose tolerance than others and maintained levels of blood glucose under 8.27 ± 0.16 mmol/l in 72 h. $AUC_{glucose}$ also proved that liraglutide, exenatide or the GLP-1/peptide 2 complex did not exhibit glucose regulatory activity 72 h after single dose injections, but the rats treated with the GLP-1/peptide 1 complex showed a 1.6-fold decrease in glucose levels at 72 h, $P < 0.01$ (Fig. 6B). The single dose injection glucose tolerance test suggested that the GLP-1/peptide 1 complex exerted a long-lasting glucose regulatory effect in rats.

IPGTT was also performed to assess whether the GLP-1/peptide 1 complex has an anti-diabetic effect during long-term administration in ZDF rats. The results in Fig. 7A indicated that ZDF rats injected with the GLP-1/peptide 1 complex, exenatide and liraglutide presented blood glucose levels lower than the control rats (lean ZDF rats injected with saline). The lower concentration of blood glucose was observed in these groups 2 days after the cessation of drugs. The $AUC_{glucose}$ of treated groups were statistically lower than that of the control group ($P < 0.01$, Fig. 7B). $HbA_{1c}$ results indicated that the GLP-1/peptide 1 complex improved the glucose tolerance of rats, as with exenatide and liraglutide. During the 28-day treatment, the $HbA_{1c}$ of the control group was 8.2% ± 0.18% (66 mmol/mol). The $HbA_{1c}$ of GLP-1/peptide 1 treated animals decreased to 7.0% ± 0.15% (53 mmol/mol), whereas liraglutide reduced $HbA_{1c}$ to 7.37% (57 mmol/mol), and exenatide reduced $HbA_{1c}$ to 7.51% ± 0.17% (59 mmol/mol), respectively. Efficient glucose tolerance and $HbA_{1c}$ reduction demonstrated that the GLP-1/peptide 1 complex possesses an anti-diabetic effect.

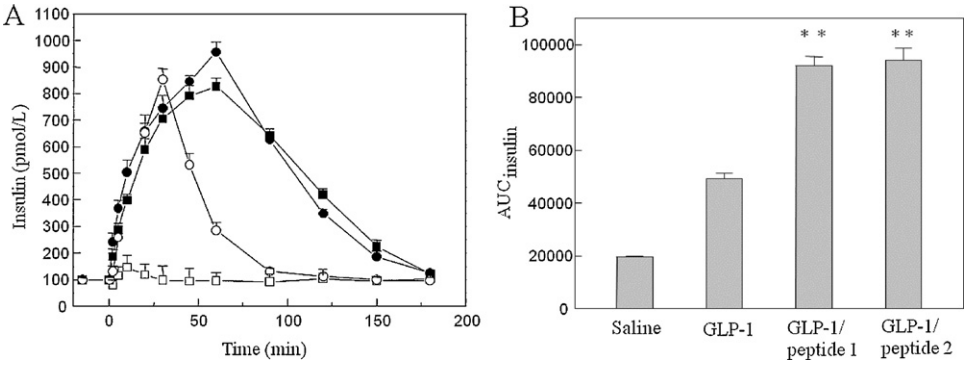


**Fig. 4 – Insulin response of GLP-1/peptide complexes treated SD rats upon an oral glucose administration. Panel A. Stimulation of insulin secretion by GLP-1/peptide complexes in SD rats. Conditions: GLP-1/peptide 1 complex (1:10, containing GLP-1 100 μg) (●) or GLP-1/peptide 2 complex (1:10, containing GLP-1 100 μg) (■) were injected intraperitoneally into SD rats; glucose was taken orally (10 g/kg). The concentration of insulin was measured by insulin ELISA kit. Amount of 100 μg GLP-1 (○) or saline (□) was injected in control group. Data are means ± SE, *n* = 6. Legend: result indicated that GLP-1/peptide complexes stimulated insulin secretion *in vivo*, and had long-acting insulin regulatory. Panel B. $AUC_{insulin}$ upon the oral glucose administration. Legend: $AUC_{insulin}$ calculated from the injection of glucose to the end of the test (180 min) showed that the level of insulin in Sprague Dawley rats injected by GLP-1/peptide complexes increased 2-fold than that of rats injected by GLP-1, $^{**}P < 0.01$.**

## 4. Discussion

The known physiological functions of GLP-1 include the increase of insulin secretion from the pancreas in a glucose-dependent manner [23], the decline of glucagon secretion from the pancreas [9], the increase of β cell mass and insulin gene expression [24], the inhibition of acid secretion and gastric emptying in the stomach [25], and the decrease of food intake by increasing satiety [26]. These findings imply that GLP-1 plays a critical role in the regulation of glucose homeostasis, suggesting that GLP-1 is a feasible candidate in the treatment of type 2 diabetes mellitus. Despite its attractive anti-diabetic action, the therapeutic potential of using native GLP-1 is limited by its short lifetime (<2 min) *in vivo*, which is mainly due to rapid enzymatic inactivation by DPP-IV [27] and quick renal clearance within 10 min [28]. It is therefore essential to overcome this half-life obstacle for the clinical utility of human GLP-1.Protecting peptide containing hydrophilic and hydrophobic domains was considered to increase the half-life of GLP-1 *in vivo* [20,29]. Protecting peptide was presumed to form a stable complex by hydrophilic interactions and build a shell to protect GLP-1 against enzymatic degradation [21]. We report here that a stabilized complex containing GLP-1 and a protecting peptide retains the glucose regulatory property and possesses long-acting characteristics. Our *in vitro* and *in vivo*

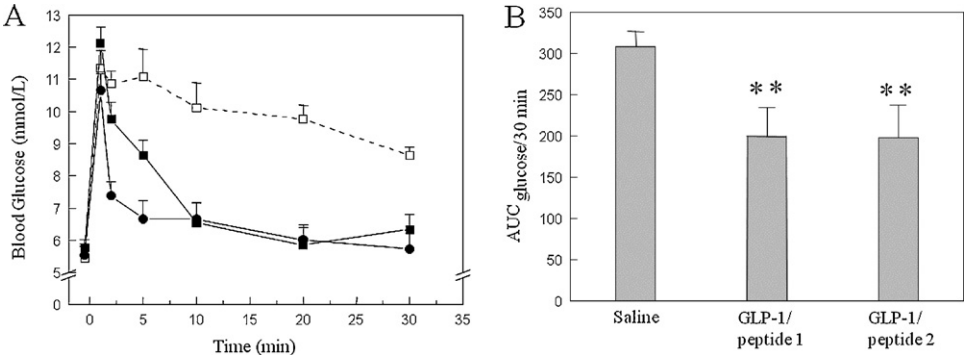


**Fig. 5 – Glucoregulatory effects of GLP-1/peptide complexes. Panel A. The effect of GLP-1/peptide complexes on the level of blood glucose upon glucose administration. Conditions: SD rats were i.p. injected with GLP-1/peptide 1 (●) (GLP-1 ~100 μg/kg body weight) or GLP-1/peptide 2 (■) (GLP-1 ~100 μg/kg body weight) after 16 h fasting. Glucose (2 g/kg) was administrated intraperitoneally at 30 min afterwards. Concentrations of blood glucose were measured at 2, 3, 5, 10, 20 and 30 min after glucose administration. Saline (□) was injected in control group. Legend: result showed that blood glucose decreased significantly upon the administration of both GLP-1/peptide complex and GLP-1/peptide 2 complex in 30 min. Within the initial 5 min, GLP-1/peptide 1 showed quicker glucose decrease than GLP-1/peptide 2 complex. Panel B. $AUC_{glucose}$ of 30 min glucose curve. Legend: AUC showed that the concentration of blood glucose in SD rats treated with GLP-1/peptide 1 or GLP-1/peptide 2 decreased 35% than the control, $^{**}P < 0.01$.**

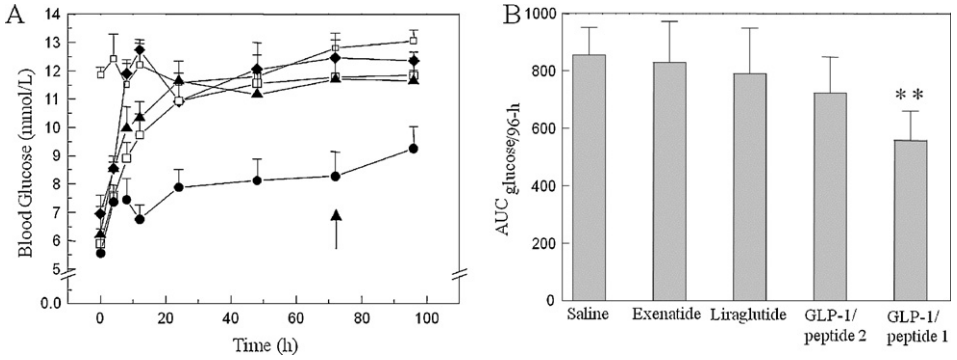


**Fig. 6 – Glucose tolerances of GLP-1/peptide complexes in single dose test. Panel A. The effect single injection of GLP-1/peptide complex on glucose regulation. Conditions: fasting SD rats were intraperitoneally injected of GLP-1/peptide 1 (solid circle) (GLP-1 ~300 μg/kg body weight), GLP-1/peptide 2 (solid square) (GLP-1 ~300 μg/kg body weight), Liraglutide (solid triangle) (100 μg/kg body weight) and Exenatide (solid diamond) (300 μg/kg body weight). Glucose (2 g/kg body weight) was administrated at 30 min before each time point (0–96 h). Saline (cored square) was injected as a control. Legend: the administration of GLP-1/peptide 1 complex maintained the concentration of blood glucose in normal level for 72 h (arrow) upon the single dose injection; other groups had an extremely short glucose tolerance (4–8 h). Panel B. $AUC_{glucose}$ of area under 96 h glucose curves. Legend: the calculated $AUC_{glucose}$ within 96 h had a 35% decrease in the rats treated with GLP-1/peptide 1 complex, $^{**}P < 0.01$.**

stability assay results support the notion that GLP-1 was still detectable 5 days after a single dose injection in rats (Fig. 3). The present study confirmed that the complex of GLP-1 and protecting peptide 1 potentiates glucose stimulated insulin secretion *in vivo*, produces a significant dose dependent increase in insulin secretion after the glucose challenge, and prolonged insulin regulation (Fig. 4). The increased insulin level together with the prolonged secretory response of insulin secretion observed in the Sprague Dawley rats resulted in much greater insulin levels over the course of the experiment (180 min). The results also demonstrated that the protecting peptide did not retard the biological effects of GLP-1 (Fig. 5).

To assess the anti-diabetic effect of GLP-1/peptide 1, we performed glucose tolerance tests with single dose injections and long-term glucose tolerance tests in animals. IPGTT data displayed that the glucoregulatory effect of the GLP-1/peptide 1 complex was preserved for 4–5 days after a single dose injection, indicating that the detected complex was still biologically active (Fig. 6). The single dose injection test demonstrated that the glucose tolerance of GLP-1/peptide 1 in 5 days is better than that of exenatide and liraglutide. The mean blood glucose level was reduced remarkably during treatment with GLP-1/peptide 1 complex within 28 days, as judged by $HbA_{1c}$ determination (Fig. 7). Additionally, the protecting peptide did not possess any anti-diabetic effects in this study (data not shown). Our study

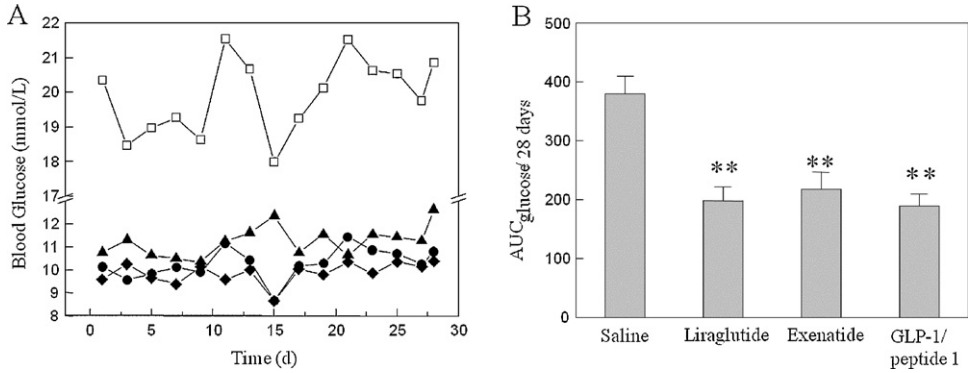


**Fig. 7 – Glucose tolerances of GLP-1/peptide complexes in multiple dose test. Panel A. Long-lasting glucose regulatory effect of GLP-1/peptide 1 complex in ZDF rats. Conditions: GLP-1/peptide 1 complex (solid square) (300 μg GLP-1/kg body weight) was administrated every 4 days during the whole experimental period of 28 days. Liraglutide (solid triangle) (300 μg/kg body weight) was administrated daily and Exenatide (solid diamond) (100 μg/kg body weight) was intraperitoneally injected twice daily. Saline (cored square) was injected in control group. A glucometer was used to measure the level of glucose at interval time. Legend: results indicated that the GLP-1/peptide 1 treated ZDF rats maintained relatively constant lower glucose level than the control; exenatide and liraglutide showed similar glucose regulatory effect with GLP-1/peptide 1. Panel B. The level of glucose was expressed as $AUC_{glucose}$. Legend: $AUC_{glucose}$ calculated from the injection of glucose to the end of the test (28 days) showed that SD rats treated with GLP-1/peptide 1 had a 50% decrease in glucose level, when compared with control rats, $^{**}P < 0.01$.**

also demonstrated that the GLP-1/peptide 1 complex had a higher reduction of $HbA_{1c}$ than exenatide and liraglutide, suggesting that the GLP-1/peptide 1 complex could be involved in clinical development in the future.

Except Liraglutide, CJC-1131 is another GLP-1 analog that consists of a DPP-IV-resistant form of GLP-1 joined to a reactive chemical linker group that allows GLP-1 to form a covalent and irreversible bond with serum albumin following subcutaneous injection. In humans, CJC-1131 has a half-life of approximately 10 days. A 12-week, randomized, double-blind, placebo-controlled study of the effect of a combination of CJC-1131 and metformin on glycemic control in type 2 diabetic patients not achieving adequate glycemic control on metformin alone (1.5–2.25 g) or metformin plus a sulfonylurea. After 12 weeks of treatment, mean $HAb_{1c}$ values decreased by $1.1 \pm 0.23\%$ and $0.6 \pm 0.25\%$ from baseline for high- and low-dose CJC-1131-treated subjects, respectively.

In summary, we developed a novel GLP-1 application to achieve long-acting functionality and high efficacy. Base on these results, the GLP-1/peptide 1 complex was proven to improve pharmacokinetic and pharmacodynamic profiles. The GLP-1/peptide 1 complex exerted GLP-1 actions, including the stimulation of insulin secretion from the β cells and glucoregulatory and anti-diabetic effects *in vivo*. We provide a safe and efficient approach for pre-clinical and clinical research of native GLP-1.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.diabres.2011.05.021.

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