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RESEARCH PAPER

Acylation of Exenatide by Glycolic Acid and its Anti-Diabetic Activities in db/db Mice

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ABSTRACT

Purpose To prepare acylated exenatide analogues and investigate their biological properties for guiding the development of PLGA formulations of exenatide.

Methods The acylated exenatide analogues were prepared by reaction with glycolic acid (GA), one constitutional unit of PLGA, and characterized by HPLC-MS/MS and Circular Dichroism (CD). The pharmacokinetic properties and anti-diabetic activities were studied in SD rats and db/db mice, respectively.

Results Structural characterizations of the acylated products showed that one to four glycolic acids (GAs) were connected to the primary amine groups of exenatide, and there was a conversion of α-helix to β-sheet to some extent. Pharmacokinetic studies in SD rats revealed that acylated exenatides had a similar Tmax with that of the prototype drug, whereas the Cmax and the AUC values of the adducts were significantly decreased. Biological activity tests demonstrated that exenatide and acylated exenatide analogues had similar *in vivo* antidiabetic activities in terms of controlling blood glucose concentration, HbA1c level, body weight and food intake.

Conclusions These findings suggest that GA conjugated exenatide had no influence on the peptide efficacy, therefore it's not necessary to inhibit exenatide acylation in PLGA formulations during the peptide release process.

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KEY WORDS acylation · anti-diabetic activity · exenatide · glycolic acid · stability

ABBREVIATIONS

CD	Circular dichroism
CDI	N, N′-carbonyldiimidazole
DMSO	Dimethyl sulfoxide
GA	Glycolic acid
LCA	Lithocholic acid
LUA	Lauric acid
PLGA	Poly(lactide-co-glycolide)
SD	Sprague–Dawley
T2DM	Type 2 diabetes mellitus

INTRODUCTION

The incidence of type 2 diabetes mellitus (T2DM) is increasing rapidly in the whole world now. The prevalence of this disease was 171 million in 2000 globally, and this data will be 366 million in 2030 (1). In type 2 diabetes treatment, incretin-based therapeutics is now becoming more and more important, and its place is now being recognized by more and more clinical endocrinologists (2). Among this kind of drugs, exenatide (synthetic exedin-4) is an analogue of Glucagon-like peptide-1 (GLP-1) sharing more than 50% homology (3). Due to its high potency in glucoregulation, such as causing the release of insulin from pancreatic β cells and suppressing the secretion of glucagon from the pancreas, exenatide (Byetta®) has recieved the approval of US Food and Drug Administration (FDA) in 2005 and is indicated for therapy of patients with T2DM.

Although the biological half-life of exenatide is a longer than that of GLP-1 (2.4 h *vs.* less than 5 min in humans) in resisting enzymatic degradation caused by dipeptidyl peptidase IV (DPP IV), one subcutaneous injection of this peptide

cannot maintain effective glucose control for more than 8 h (4). To increase therapeutic effects as well as convenience and compliance of patients, improvement of the dosing from bid (twice daily) to a sustained release formulation will be of practical benefit (5–7). The sustained release microsphere of exenatide (Bydureon™) developed by Amylin has been approved by EMEA and FDA in June, 2011 and in Jan, 2012, repectively. In this formulation, the biodegradable poly(lactide-co-glycolide) (PLGA) acts as the matrices controlling the release of the peptide from the dosage form for at least 2 weeks.

In general, the sustained release microspheres will remain in the *in vitro* release medium or at the *in vivo* injection site from several weeks to several months, and the high temperature and the humid environment thereof will present a great threat to the stability of the incorporated drugs during the release process. Like many other peptides, exenatide can also form acylated adducts due to the interaction between the amine groups of the peptide and the ester groups of the polymer (8). Although there are many reports about the mechanism of peptide acylation (8–10) as well as the preventing strategies of this reaction (11–18), the reports on the biological properties of these acylated peptides are very few (19). Acylation of peptides and proteins by some acid anhydrides were reported to have some unwanted effects, for instance, a loss of activity (20,21) or a change of receptor specificity (22,23) and immunogenicity (21,23). These cases, however, do not represent what associated with PLGA and PLA polymers.

It can be well understood that, for small short peptides, acylation is not a good precursor, as it may interfere the receptor binding of the drug as well as its efficacy. Before the substituents are removed, the peptide may have already been degraded in the *in vivo* environment. However, for the peptides and proteins with special spatial structures, there may be little or no influence of acylation on the efficacy of the drug if the acylated residues do not hinder the receptor binding. If the degradation products have no clinically relevant adverse effects, they can be qualified [ICH (11/2005) Guideline for industry: Q5C: Quality of biotechnological products: Stability testing of biotechnological/biological products. ICH (06/2006) Guidance for industry: Q3B(R2): Impurities in new drug products]. Therefore, it should be a case-by-case study on these byproducts, and inhibition of acylation without assessment on their biological consequences seems not reasonable and somewhat pointless.

In this study, the acylated exenatide was prepared by reaction with activated glycolic acid and characterized by HPLC-MS/MS and circular dichroism (CD). The pharmacokinetic tests of acylated exenatide analogues were performed in male Sprague–Dawley (SD) rats, and the *in vivo* anti-diabetic activities of the acylated peptides were examined in *db/db* mice to investigate the influence of peptide acylation on the biological efficacy. The tests of exenatide were carried out at the same time for comparison.

MATERIALS AND METHODS

Materials and Animals

Exenatide was purchased from Soho-Yiming Pharmaceuticals Co., Ltd. (Shanghai, China); glycolic acid (GA) was from Sigma Aldrich Co. (USA); N, N′-carbonyldiimidazole (CDI) was from Alfa Aesar Chemistry Co., Ltd. (Shanghai, China); all other chemicals used were of reagent grade.

Sprague–Dawley (SD) rats (male, body weight of 220-260 g) were obtained from the Experimental Animal Center of Shandong Luye Pharmaceutical (Yantai, China) and used in the pharmacokinetic study. Type 2 diabetic *db/db* mice (BKS.Cg-Dock7m +/+ Leprdb/J, male, 14 weeks old) were purchased from Jackson Lab (Nanjing, China). All studies were performed in conformity with 'Principles of Laboratory Animal Care' (NIH publication no. 85–23, revised 1985) and Experimental Animal Research Committee at Yantai University.

Preparation and Characterization of GA Acylated Exenatide

Exenatide acylations were performed using coupling reactions between N, N′-carbonyldiimidazole (CDI) activated glycolic acid (GA) and the amine groups of exenatide in DMSO : water solution. Briefly, 30 ml of GA-CDI (3.16 mg/ml GA mixed with 6.75 mg/ml CDI for 30 min in DMSO) was mixed with 20 ml of exenatide (2.63 mg/ml in deionized water) (exenatide/GA-CDI molar ratio of 1/100) at room temperature for 2 h. The solution was then dialyzed in dialysis bag (2 K MWCO) for 24 h and freeze-dried to get the final acylated products.

The detection of the acylated peptides was performed by HPLC using an analytical column (Jupiter XB-C18, 4.0 mm × 250 mm) from Phenomenex (Torrance, USA). A gradient elution was performed with a mobile phase A (double-distilled water/acetonitrile (80/20) + 0.5% v/v H_3PO_4) and a mobile phase B (acetonitrile + 0.5% v/v H_3PO_4). The eluent varied from 85 to 71% A in 17 min in a linear way with a flow rate of 1.0 ml/min. Chromatograms were recorded at 210 nm (UV detector). Exenatide standards were used for comparison.

The identification of the acylated peptides was carried out on a mass spectrometer (API 4000, Applied Biosystems Sciex, Ontario, Canada) using an ESI source in the positive ion mode, and the Q1 scan mode was in the range of 500–1500 amu, as previously described (24,25). Briefly, optimized conditions of MS were as follows: Curtain gas: 10 units, gas 1: 45 units, and gas 2 (all nitrogen): 40 units; ion spray voltage:

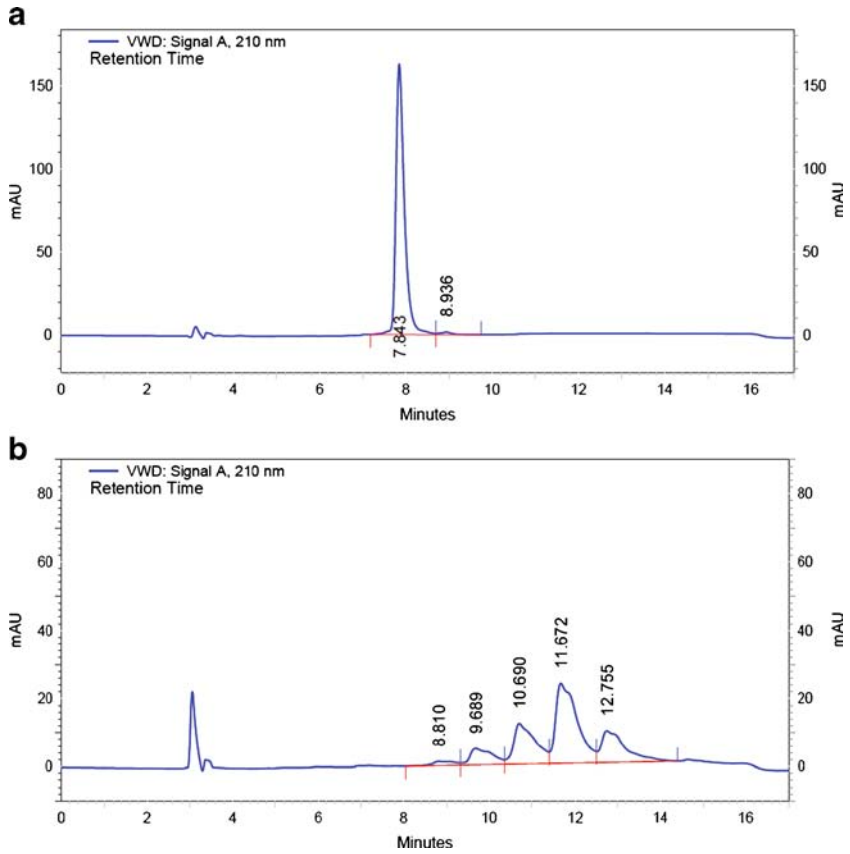

Fig. 1 The HPLC characterization of the peptide adducts. (**a**) Exenatide. (**b**) Acylated exenatide analogues by glycolic acids.

5500 V; source temperature: 400°C; declustering potentials: 50 V. The mobile phase was based on a linear variation of 30–44% solvent B (acetonitrile +0.01% v/v TFA) in solvent A (double-distilled water +0.01% v/v TFA) over 20 min with a 1 ml/min flow rate. The samples (about 10 μl) were separated on a C18 reversed phase analytical column (Jupiter XB-C18, 4.0 mm × 250 mm) from Phenomenex (Torrance, USA). The Analyst software was utilized for data processing.

Circular Dichroism (CD) Spectroscopy

Far-UV CD spectra were recorded on a Jasco J-810 spectropolarimeter (JASCO Inc., Easton, MD) at 20°C in a 1 cm quartz cuvette. The scanning range was from 190 to 260 nm (20 nm/min, bandwidth of 1 nm). The concentration of both exenatide and the acylated products was 0.01 mg/ml in triple distilled water. The spectra were corrected for background contributions and converted to mean residue ellipticity (MRE, deg•cm^2 dmol^{-1}) by the following equation (Eq. (1))

$$\mathrm{MRE} = \left(\frac{\theta \times MW}{10 \times r \times c \times L}\right) \quad (1)$$

Where θ is the observed CD signal (units of millidegrees), Mw is the molecular weight of the peptide, c is the concentration (mg/mL), r is the number of amino acids residues, and L is the path length. For exenatide, values of 4186.6 and 39 were input for Mw and r, respectively. For the acylated products, an average Mw of 4364.1 was used in the calculation. Deconvolution of the CD data was carried out using CD Pro software.

Pharmacokinetics in SD Rats

Pharmacokinetic studies on exenatide and acylated exenatide were performed in male SD rats. Exenatide and acylated exenatide were dissolved in double distilled water (20 μg/ml). Rats (n=4 each group) were administered s.c. with the peptide and the acylated peptide, respectively. The samples were taken at predetermined time points for the solution injection and centrifuged at 3000 rpm, and the plasma obtained was kept at −40°C before analysis. The determination of drug concentration was performed with ELISA using commercial Exendin-4 Fluorescent EIA kits under the instructions supplied by the manufacturer.

Antidiabetic Effects in *db*/*db* Mice

The antidiabetic activities of acylated exenatide were assessed in BKS.Cg-Dock7m +/+ Leprdb/J *db*/*db* mice (14 weeks old male) after grouping according to the fasting glucose, body

weight and HbA1c levels. Under non-fasting conditions, mice received a single s.c. administration of saline, exenatide (10 nmol/kg), and acylated exenatide (10 nmol/kg) on day 0, respectively. Blood glucose levels were measured on the samples from tail-vein blood with ACCU-CHEK Active (Roche Diagnostics, Germany) at 0, 1, 2, 4, 6, 8 and 24 h

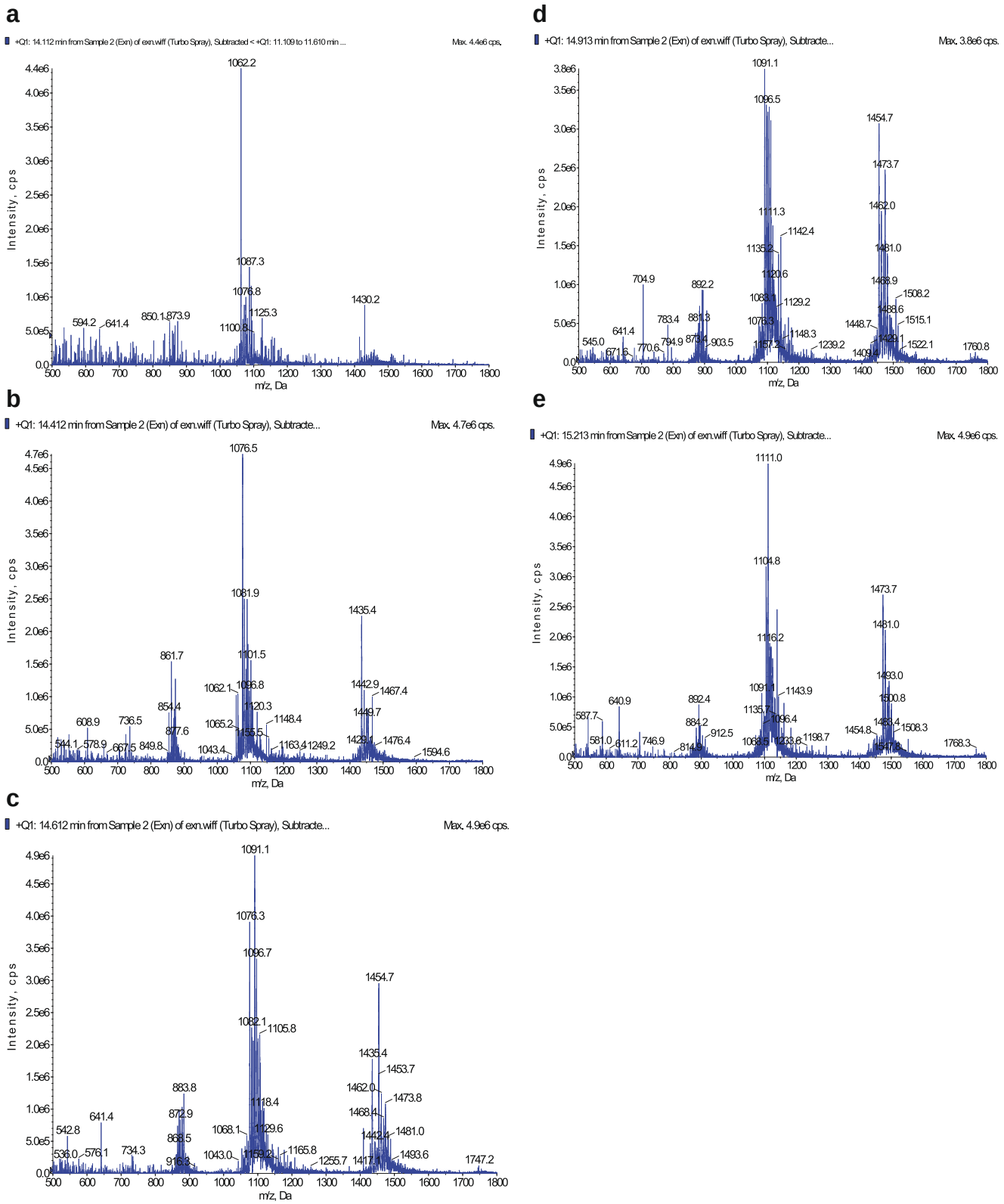

Fig. 2 Electrospray mass spectrum of acylated exenatide analogues. (**a**) Exenatide-GA. (**b**) Exenatide-2GA. (**c**) Exenatide-3GA. (**d**) Exenatide-3GA. (**e**) Exenatide-4GA.

after injection. From day 1 to day 15, mice received twice-daily s.c. administration of saline, exenatide (10 nmol/kg), or acylated exenatide (10 nmol/kg) at 9:00 a.m and 16:30 p.m., respectively. Blood glucose levels were measured at 2 h after administration on days 1, 4, 8,11 and 15 for all groups.

Body weight and cumulative food intakes were also determined during the long-term pharmacodynamic test to monitor the appetite alteration.

HbA1c levels in blood samples obtained from the tail-vein (about 10 μl) were determined for all groups on day 0 and day 15 using a DCA™ Vantage analyzer (SIEMENS Healthcare Diagnotics Ltd. UK).

Statistical Analysis

All results are reported as means ± SDs (*in vivo* experiments). Statistical analyses on group comparisons were performed with the Student's *t*-test. $P<0.05$ was considered of significance, and $P<0.01$ was considered of high significance.

RESULTS

Preparation and Characterization of GA Acylated Exenatide

To simplify the preparation of acylated exenatide, the reaction was performed in DMSO-water solution with glycolic acid activated by CDI as the electrophile. RP-HPLC and HPLC-MS/MS were used to examine the formation of the acylated exenatide. Figure 1a and b show the HPLC chromatograms of exenatide before and after incubation with activated glycolic acid. The intact exenatide peak was at a retention time of 7.843 min. The acylated products peaks appeared at retention time of 8.810–12.755 min, and analyzed by HPLC-MS/MS (Fig. 2a–e). Table 1 shows the mass spectrum data of these peaks, which are assigned to exenatide-GA, exenatide-2GA, exenatide-3GA and exenatide-4GA. The average molecular weight of the acylated mixture was calculated to be 4364.1 according to the peak areas, which means the average molecular weight increase was about 4.2%.

Circular Dichroism Spectroscopy of GA Acylated Exenatide

Far-UV CD spectroscopy was used to examine the secondary structure of exenatide and acylated exenatide analogues. The tests were carried out with a low concentration of the peptides, so that both exenatide and the acylated products could exist as monomers and decrease the interference of peptide aggregation (26,27). The far-UV CD spectra of exenatide had two strong negative bands at 209 and 224 nm with a positive band at 254 nm. For acylated exenatides, a significant decrease in the 209 and 224 nm bands was seen in the spectra, implying a change of the peptide secondary structure (Fig. 3). These alterations were quantitated using CD Pro software (Table 2), and the data shows that the peptide underwent a transition of α-helical structure to β-sheet structure.

In Vivo Pharmacokinetics of Exenatide and Acylated Exenatide

In vivo pharmacokinetic studies were carried out using exenatide and acylated exenatide in male SD rats. The plasma profiles after s.c. administration are shown in Fig. 4. In SD rats injected with exenatide at a dose of 20 μg/kg, the plasma concentration of the peptide increased rapidly after injection, reached the peak within 1 h (T_{max} of 0.5 h, C_{max} of 8.612 ng/ml and $AUC_{0\text{-}24h}$ of 12.49 ng/ml•h), and followed by a rapid decrease to baseline after 4 h. In the group treated with acylated exenatide at the same dose, a significantly lower plasma concentrations were observed with a T_{max} of 0.5 h, C_{max} of 3.783 ng/ml and $AUC_{0\text{-}24h}$ of 8.37 ng/ml•h. The differences in the plasma concentration between exenatide and the acylated products may be ascribed to the limitations of the ELISA assay, where there is a competitive binding of the biotinylated peptide supplied by the manufacture of the kits with the substrate of samples to the peptide antibody (primary antibody). The substituents of GA, especially at the N-terminus, namely the His^1 residue, may decrease the epitope for the primary antibody in the ELISA (28), and thus interfere the binding of the acylated samples therein and result in a lower data.

Table I Characterization of the Acylated Products of Exenatide by ESI-3Q MS

HPLC peak	Retention time (min)	Area percent (%)	Observed m/z (amu)		Observed molecular mass (amu)	Expected structure
			$(M + 3H)^{3+}$	$(M + 4H)^{4+}$		
1	8.810	2.387		1062.2	4244.8	Exenatide + GA (+58 amu)
2	9.689	9.302	1435.4	1076.5	4302.6	Exenatide + 2GA (+116 amu)
3	10.690	23.284	1454.7	1091.1	4360.8	Exenatide + 3GA (+174 amu)
4	11.672	44.913	1454.7	1091.1	4360.8	Exenatide + 3GA (+174 amu)
5	12.755	20.114	1473.7	1104.8	4416.7	Exenatide + 4GA (+232 amu)

In Vivo Antidiabetic Effect of Acylated Exenatide in db/db Mice

As shown in Fig. 5a, the blood glucose levels of saline-treated db/db mice were at 28 mmol/L, while exenatide (10 nmol/kg) or acylated exenatide (10 nmol/kg) rapidly reduced blood glucose levels to 17 mmol/L after subcutaneous injection, and maintained the normal glucose levels for about 6 h. Thereafter, the glucose levels reached the hyperglycemic level (>24 mmol/L) again and maintained that state. Continuous daily injections of both exenatide and acylated exenatide into non-fasting db/db mice could adjust blood glucose levels to a normal state during the time course according to the results of the long-term test (Fig. 5b).

Figure 5c shows that the non-fasting HbA1c levels were decreased for all of the drug injected groups compared to the saline injected group at the end of this test. However, the difference between the solution formulation injected groups and that of saline injected group was of no significance, and this may be ascribed to the lack of continuous GLP-1R activation for both of the two liquid injections.

The acylated peptide also had a similar effect on the food intakes and body weight of the *db/db* mice to that of exenatide. As presented in Fig. 5d and e, there were no much difference in the body weight and food intake between the saline-treated control and that of the drug-treated groups, and all of the groups had a similar increase in the body weight to some extent during the test period.

DISCUSSION

Exenatide is amphipathic in nature (29) and will form oligonomer in aqueous solution at a concentration more than 10 μM (26). To facilitate the acylation reaction, 60% DMSO-water was used as the solvent medium where exenatide existed as monomer and had good accessibility to activated glycolic acids, and the conversion of the peptide was almost complete (24–26). Exenatide has three possible acylation sites, two lysine residues (Lys^{12} and Lys^{27}) and one primary group of the N-terminus (His^{1}), therefore the products were more complex than that formed in PLGA microspheres during *in vitro* or *in vivo* release (24). Nevertheless, the mixture can be used to investigate the effect of the peptide structure alteration on its biological properties (19).

Table 2 Calculation results of Far-UVCD of EXENATIDE and Acylated Exenatides

Sample	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coil (%)
Exenatide	24.4 ± 1.08	19.0 ± 4.48	21.0 ± 1.92	35.0 ± 4.66
Acylated exenatides	16.7 ± 3.58	26.6 ± 0.86	20.9 ± 1.87	34.5 ± 1.71

The values represent an average of data from three independent programs

The molecular weight of exenatide is 4186.6, which is significantly more than that of glycolic acid with a molecular weight of 58. Even the three amine residues are all bound by glycolic acids, the average content of the moieties is less than 5% in the conjugate. Therefore, it's not necessary to separate the mixture to get the single compound for further evaluation. However, the acylation will change the electrical properties of the amine groups due to the conversion of aliphatic amines to the carbonyl amides, what's more, these small substituents may have an influence on the higher order structure of the peptide momoner due to its high hydrophilicity according to the results of far-UV CD.

When the in vivo biological properties are taken into account, however, there were almost no difference between

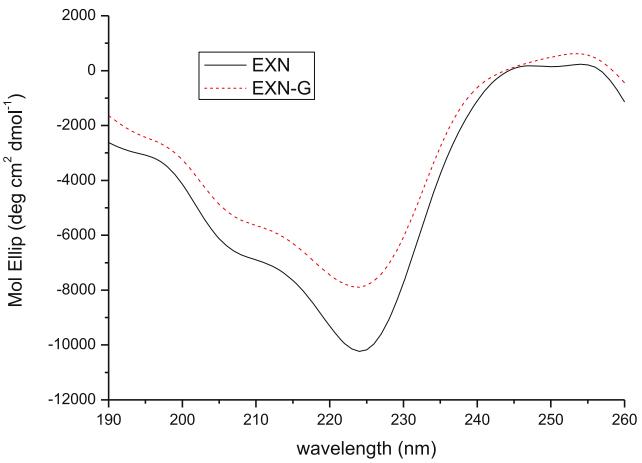

Fig. 3 Far-UV CD scans of exenatide and acylated exenatide analogues (0.01 mg/ml). *Solid line* shows spectra for exenatide. *Dashed line* shows spectra for acylated exenatide.

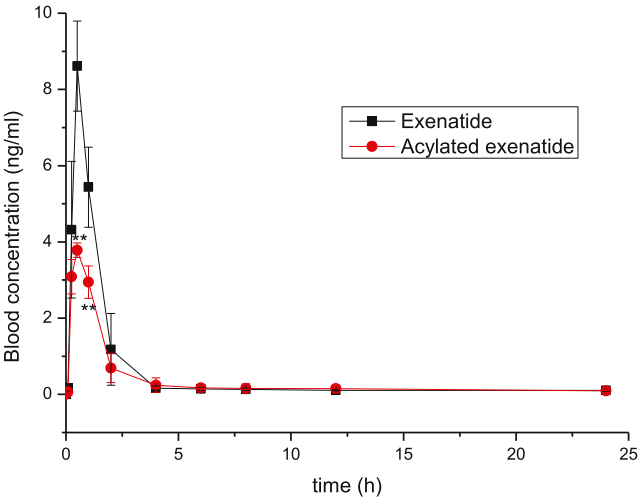

Fig. 4 Pharmacokinetic profiles of exenatide (s.c.) and acylated exenatide (s.c.) in SD rats (20 μg/ml, $n=4$ each group). *$P<0.05$ and **$P<0.01$ compared to exenatide injected group.

exenatide and its acylated products with respect to pharmacodynamics behaviors. It was reported that the multiple substitutions of LCA or FA on exenatide could cause considerable damaged insulinotropic activity and GLP-1 receptor binding ability as a result of the disturbance of the higher order structure and the increased molecular volume of the peptide, which can lead to prevention of effective GLP-1 receptor bindings (30,31). However, glycolic acid is a shorter aliphatic acid of 2 carbon atoms compared to LCA of 22 carbon atoms and LUA of 12 carbon atoms, therefore, the increase in the

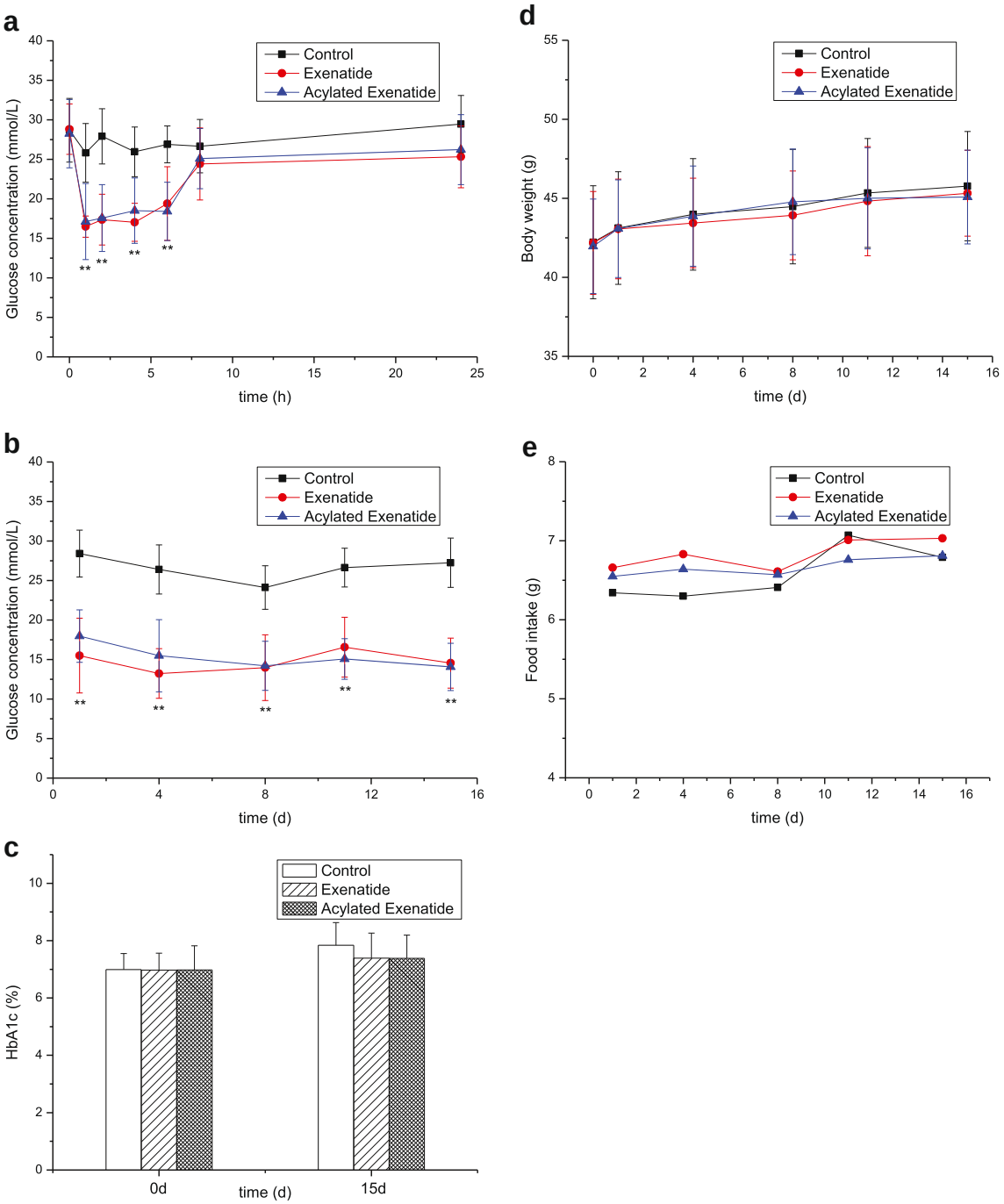

Fig. 5 The anti-diabetes effects of exenatide and acylated exenatide analogues on *db/db* mice ($n=10$). (**a**) The acute glucose regulating effects of exenatide and acylated exenatide analogues on non-fasted *db/db* mice (s.c., 10 nmol/kg, $n=10$). (**b**) The long-term glucose regulating effects of exenatide and acylated exenatide analogues on non-fasted *db/db* mice (s.c., 10 nmol/kg, twice daily, $n=10$). (**c**) The HbA1c regulating effects of exenatide and acylated exenatide analogues on non-fasted *db/db* mice after 15 days (s.c., 10 nmol/kg, twice daily, $n=10$). (**d**) Change in body weight in *db/db* mice after exenatide and acylated exenatide analogues administration for 15 days (s.c., 10 nmol/kg, twice daily, $n=10$). (**e**) Cumulative food intake in *db/db* mice after exenatide and acylated exenatide analogues administration for 15 days (s.c., 10 nmol/kg, twice daily, $n=10$). $^{*}P<0.05$ and $^{**}P<0.01$ compared to saline injected controls.

bulkiness of the molecule does not present a serious hindrance to the receptor bindings.

The *in vitro* characterization of the acylated products showed the alteration of the peptide higher order structure to some extent, however, the receptor-bound conformation may be different from that in solution for the ligands of Class B GPCRs (32). Furthermore, there are eight identical amino acids in the central region of both exenatide and GLP-1, and for exenatide, they are S(Ser)^{11}, E(Glu)^{15}, A(Ala)^{18}, F(Phe)^{22}, I(Ile)^{23}, W(Trp)^{25}, L(Leu)^{26} and G(Gly)^{29}, while for GLP-1, they are S(Ser)^{17}, E(Glu)^{21}, A(Ala)^{24}, F(Phe)^{28}, I(Ile)^{29}, W(Trp)^{31}, L(Leu)^{32} and G(Gly)^{35}. These residues lie on the same face on the helical region, which means that this face of the helix will play an essential role in the critical contact with the receptor (33). The acylation sites of Lys^{12} and Lys^{27} are close to Ser^{11} and Leu^{26}, however, they are located on the other face of the helical region, therefore, acylation of these two lysines by glycolic acids will not interfere the receptor bindings (Fig. 6).

Although it was reported that chemical modification of the molecular structure of exenatide could cause some alteration on their physiochemical and biological properties, they could not replace the microsphere formulations of exenatide in terms of improvement of efficacy and patients compliance. On the other hand, acylation may cause threats to safety and effective delivery of peptides and proteins in PLA and PLGA dosage forms, what's more, this reaction would not be discovered when the sealed drug vials were placed under controlled conditions of humidity and temperature, but it could occur in the solution medium during the *in intro* release test, or in the injection sites during the *in vivo* biological test (9,34). However, there are no significant difference in anti-diadetes activities for exenatide and its acylated products according to our experiments. Furthermore, since glycolic acid and lactic acid are biodegradable and cleared by the Krebs cycle (35), it can be inferred that these components will not cause severe issues on safety, though acylation itself may affect the release behavior of peptides and proteins from the extended release dosage forms based on PLA and PLGA (36,37). Therefore, it's not necessary to add stabilizing or blocking agents to suppress exenatide acylation in PLGA microspheres from the point of view on anti-diabetic activities at least.

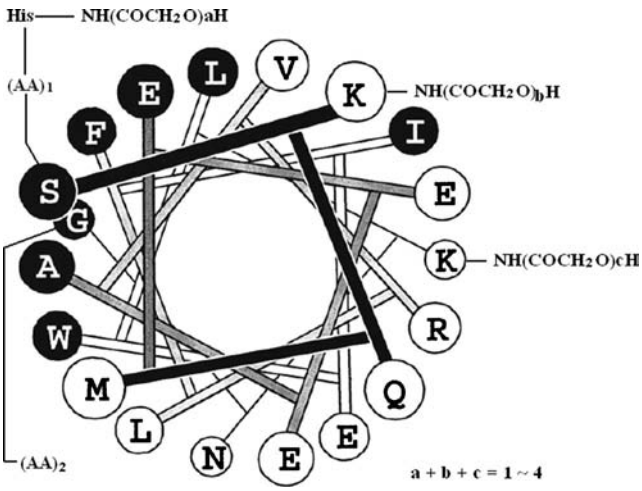

Fig. 6 The structure of acylated exenatide. The eight identical residues in both GLP-1 and exenatide are shown in *white lettering* on a black background in the helical wheel. $(AA)_1$ is fragment of -Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu- in the N-terminus, and $(AA)_2$ is fragment of -Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2 in the C-terminus (adapted from López de Maturana and Donnelly's work with the authors' permission).

CONCLUSION

Our findings indicate that acylation reaction would not affect the anti-diabetic efficacy of exenatide in terms of lowering glucose concentrations and HbA1c levels. Therefore, it's not necessary to inhibit acylation reaction of exenatide in PLGA microspheres.

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