DOI: 10.1002/cmdc.200800053

Conjugation of ATIII-Binding Pentasaccharides to Extend the Half-Life of Proteins: Long-Acting Insulin

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Tight control over plasma glucose levels in the treatment of insulin-dependent type 1 diabetes mellitus (T1DM) can be achieved by the exogenous administration of insulin. A natural overall physiological profile can be mimicked by the administration of short- and intermediate-acting analogues to control post-meal glucose excursions and a longer-acting insulin product to maintain basal levels of insulin.[1] The very short plasma half-life of insulin poses a challenge for creating sufficiently long-acting formulations or analogues with optimal pharmacokinetic/dynamic (PK/PD) properties that provide consistent glycemic control. Approved long-acting insulin analogues for basal insulin therapy form a subcutaneous depot at the injection site (insulin glargine,[2] detemir[3]) and/or exhibit an extended half-life through hydrophobic interaction of fatty acid groups with serum albumin (insulin detemir). Both detemir and glargine require twice-daily administration.[4] Several groups have reported[5] recently on novel insulins with extended duration of action, yet there is still a need for alternatives with minimized risk of (mainly nocturnal) hypoglycemia and optimal duration of action.

An expanding repertoire of methods to enhance the exposure of protein therapeutics is emerging.[6] For instance, (bio)-chemical modification (e.g. PEGylation,[7] acylation,[8] or glycosylation[9]), fusion with or binding to plasma proteins (e.g. albumin,[10] transferrin,[11] or Fc fragments[12]) has yielded numerous clinical protein candidates with improved PK/PD profiles. The binding of a polypeptide drug (either covalently or through a tightly binding carrier) to long-lived plasma proteins in the bloodstream enables tight control over targeted drug levels with improved exposure and distribution in the circulation that may potentially lead to lower doses, decreased side effects, and enhanced predictability. In this context, not all plasma proteins may have been explored to the full extent. Herein we demonstrate how binding to the plasma protein antithrombin III (ATIII) can be used to enhance the half-life of insulin.

ATIII is a serine protease inhibitor that interrupts the blood coagulation cascade upon activation by glycosaminoglycans such as heparin, and is present in blood plasma at high concentration (~3 μM).[13] The minimal structural requirement for ATIII affinity is a sulfated pentasaccharide (PS), the fully synthetic equivalent of which reached the market in 2002 (fondaparinux, Arixtra, Figure 1).[14,15] Interestingly, the high specificity results in a much longer plasma half-life (~15 h vs. ~1 h for heparin) which can be further extended by enhancing the affinity for ATIII.[16] The latter has resulted in the anticoagulant idraparinux (K_d = 1 nM), which is now in phase III clinical development for once-weekly dosing ($t_{1/2}$ ~120 h).[15,17] In addition, the design[15] of dual-acting antithrombotics that consist of a low-molecular-weight thrombin[18] or GPIIb/IIIa inhibitor[19] conjugated to the nonreducing end of a PS has revealed that the half-life of the PS largely determines the half-life of the entire PS conjugate.

It occurred to us that the near linear PK behavior of ATIII-bound PS may be extended to conjugates of therapeutically relevant (non-anticoagulant) polypeptides and proteins that suffer from short plasma half-lives (see Figure 2). To avoid any undesired clinically significant ATIII-mediated anticoagulant activity, the polypeptide conjugate should have a therapeutic plasma level of < 50 nM (i.e. < 2% of ATIII is bound to conjugated PS, and the associated anticoagulant activity is of subtherapeutic level relative to the pharmacological activity of the polypeptide).[20] We selected insulin as an initial target to validate this concept because: a) T1DM patients require repeated injections of long-acting (basal) insulin to complement endogenous hormonal levels in the sub-nanomolar range, b) the insulin receptor (InsR) is accessible from the circulation, and c) site-directed chemical modification of insulin does not necessarily have a deleterious effect on the biological activity.[21]

Herein we disclose our novel CarboCarrier™ technology for enhancing PK/PD profiles of small proteins and polypeptides at sub-anticoagulant concentrations, exemplified by the preparation and pharmacological evaluation of novel long-acting insulins conjugated to different ATIII-binding carrier PSs with varying half-lives.

The N-terminal PheB1 and near C-terminal LysB29 amino functions are not essential for the bioactivity of insulin,[5d] and hence it was anticipated that the introduction of a PS at either

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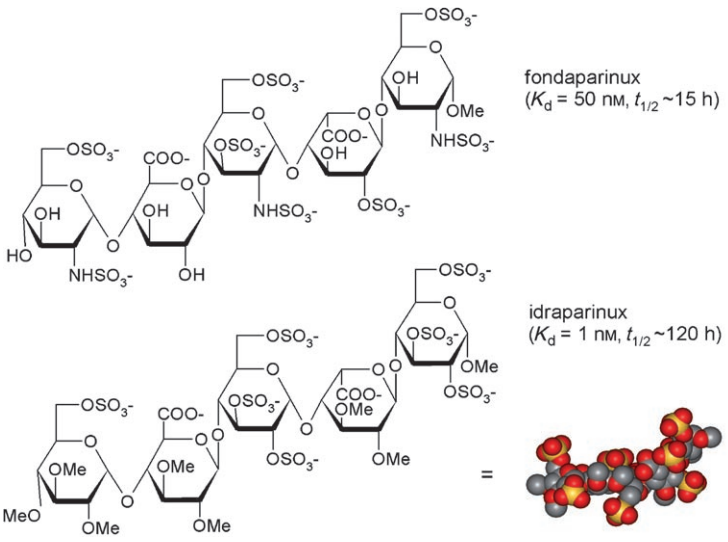


Figure 1. The ATIII-binding pentasaccharides fondaparinux and idraparinux.

of these positions would have no detrimental effect on its bioactivity. To assess whether the half-life of the insulin conjugates can be tuned by altering the affinity for ATIII, three different carrier PSs (PS^1, PS^2, or PS^3)[22,23] were introduced into recombinant human (recH) insulin with a pharmacologically inactive ethylene glycol spacer using thiol–maleimide coupling[24] chemistry (see Scheme 1 and Supporting Information). Thus, the two complementary di-*N*-Boc-protected[5b,25] recH insulins **1** or **3** were treated with the succinic ester derivative of γ-maleimidobutyric acid (GMB) to give, after acidic removal of the *N*-Boc groups and purification by preparative HPLC, the B1-GMB and B29-GMB insulins **2** and **4**, respectively. Conjugation of **2** or **4** with compound **5**, **6**, or **7** was effected by in situ NH_2OH-promoted deprotection of the thiol group and overnight stirring in buffered solution at pH 7.0 under a nitrogen atmosphere. A typical conversion of ~80% was observed based on analytical HPSEC (Superdex 75) and anion-exchange chromatography (Mono Q).

Purification of the sulfated insulin glycoconjugates **8**–**11** was carried out with preparative size-exclusion chromatography on Superdex 75 equilibrated in 50 mM ammonium acetate buffer (pH 6.8). The conjugate-containing fractions, as analyzed by anion-exchange chromatography (Mono Q), were pooled and lyophilized. The recovery, as determined by UV absorbance (A_{280}), varied between 30 and 47%. Analytical HPSEC, HPLC, and anion-exchange chromatography revealed that all conjugates could be isolated in monomeric, monosubstituted forms with >95% regioselectivity.

From a pharmaceutical perspective, it is notable that these sulfoglycosylated insulin derivatives are highly soluble (up to ~50 mg mL^{-1}) owing to their acidic pI, and are stable at room temperature for at least one month without the tendency to form aggregates. The integrity of the conjugates was further established by mass spectrometry. In Figure 3 a representative electrospray mass spectrum of compound **11** is depicted in which the observed isotope distribution pattern characteristic for a $[M+5H]^{5+}$ charged signal correlates well to the theoretical isotope distribution as calculated for this particular highly sulfated and glycosylated insulin derivative.

All target insulin glycoconjugates and their corresponding precursors were subjected to N-terminal sequence analysis (Edman degradation).[26] In each of the cycles carried out, the B29-substituted insulin derivatives **3**, **4**, and **8**–**11** yielded equimolar amounts of both A- and B-chain amino acids to a level similar to that of the initial amounts of the conjugates. This indicates full accessibility of both N termini and thus the absence of conjugate moieties which are therefore confined to the B29 position. In contrast, only A-chain amino acids were found during N-terminal sequencing of the B1-substituted insulin derivatives **1**, **2**, and **8**, demonstrating conjugation at the B1 position with consequential blocking of Edman degradation at the N terminus of the B chain.

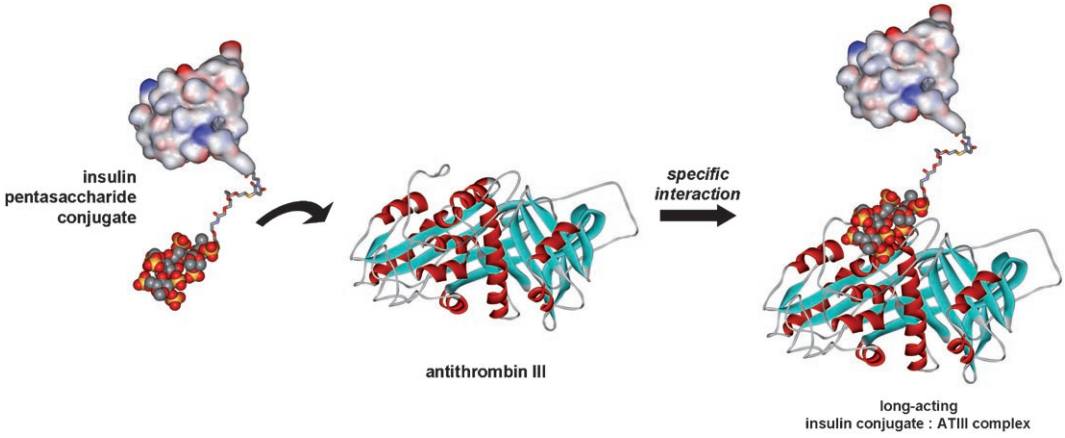
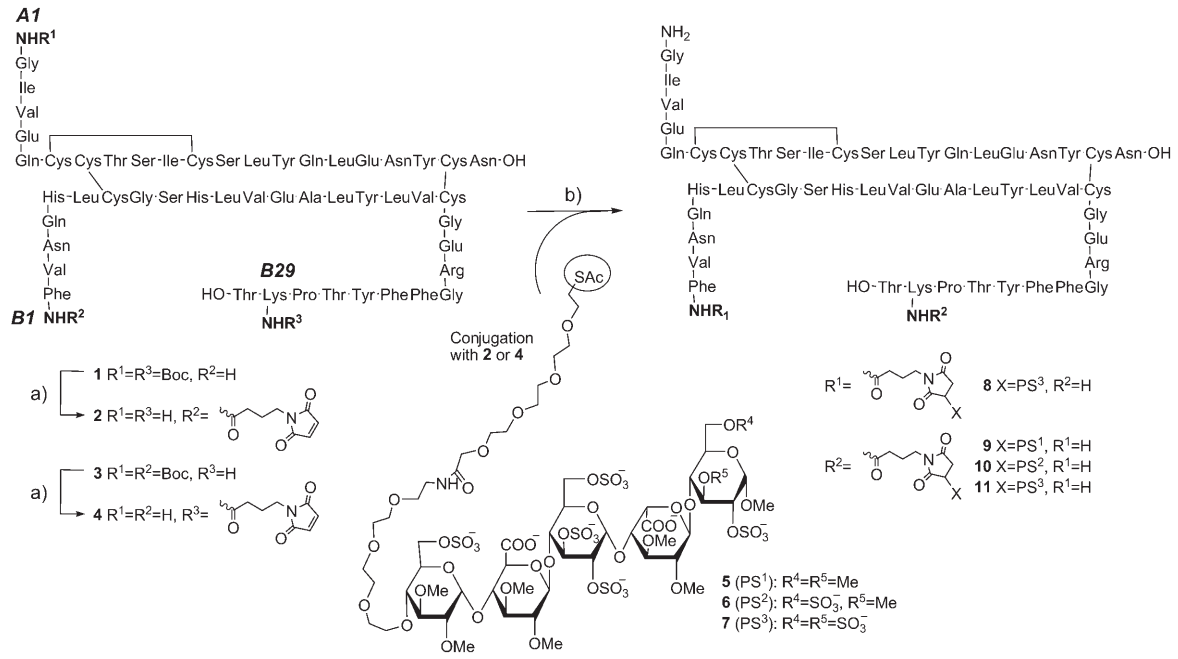


Figure 2. The short half-life of the potent (<1 nM) hormone recH insulin in plasma can be prolonged by site-selective conjugation to an antithrombin III (ATIII)-binding pentasaccharide. The specific interaction with the abundant (3 μM) plasma protein ATIII results in a prolonged duration of action.



Scheme 1. Synthesis of PS-insulin conjugates **8**–**11**. Reagents and conditions: a) 1) GMB *N*-hydroxysuccinimide ester, DMSO, Et_3N, 30 min, 2) TFA, 10 min, yield **2**: 40%, **4**: 47%; b) GMB modified insulin **2** or **4** was conjugated to **5**, **6**, or **7** using NH_2OH, 0.1 M sodium phosphate buffer, pH 7.0, 16 h, yield **8**: 47%, **9**: 33%, **10**: 30%, **11**: 45%.

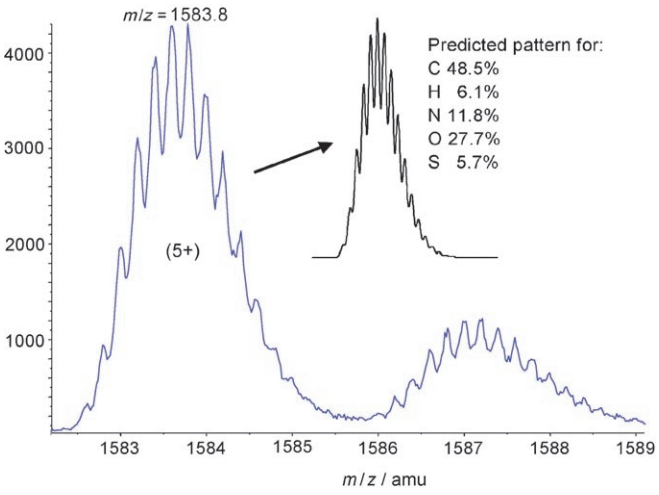


Figure 3. The experimental (blue trace) and theoretical (black trace) isotopic distribution of the most abundant charge state (the $[M+5H]^{5+}$ ion) in the ESI mass spectrum obtained from direct infusion of insulin–PS conjugate **11** into a Q-ToF mass spectrometer.

The binding affinity of conjugates **8**–**11** for hATIII was assessed by surface plasmon resonance analysis,[27] in which the potency to inhibit binding of ATIII to immobilized PS^3 was determined and compared with that of the carrier PS^1, PS^2, and PS^3 (see Supporting Information). The observed (competitive) binding potential to ATIII, expressed as IC_{50}, is different for compounds PS^1, PS^2, and PS^3 (96, 58, and 5.5 nM, respectively). Gratifyingly, the IC_{50} values of all corresponding insulin–PS conjugates (**8**–**11**) fall in the same range in comparison with their parent carrier PSs. These findings, combined with the above-described structural analysis, confirm that the binding affinity of the PS in the conjugate for ATIII is conserved and suggest that the PK properties of the conjugates can be adjusted by changing the carrier PS.

The pharmacokinetic behavior of the PS–insulin conjugates was addressed in a PK study in rats using ^{125}I-labeled compounds **8**–**11**. As depicted in Figure 4A, an enormous increase in residence time of the "long-acting" PS–insulin conjugates **8** and **11** is observed ($t_{1/2} \sim 5$ h vs. 10 min in rat). Despite this vast enhancement in circulation half-life relative to recH insulin, their PK profile does not match that of the parent PS derived from idraparinux (rat $t_{1/2} \sim 11$ h, human $t_{1/2} \sim 120$ h),[28] which is likely due to InsR-mediated clearance.[29] Nevertheless, it is anticipated that, in line with previous studies[16–18] involving PSs, the time of action of the sulfated insulin glycoconjugates **8** and **11** will allow once-a-day treatment to achieve physiological basal insulin levels in humans. The plasma clearance ($CL < 0.02\ L\,kg^{-1}\,h$) and volume of distribution ($V_{ss} < 0.10\ L\,kg^{-1}$) of the high-affinity insulin conjugates **8** and **11** were found to be decreased relative to **9** or **10** ($CL \sim 0.13\ L\,kg^{-1}\,h$, $V_{ss} > 0.50\ L\,kg^{-1}$), which have similar but moderate ATIII-binding affinities and a "medium-acting" PK profile. This substantiates the assertion that the use of a carrier PS with increased affinity for ATIII leads to an enhanced exposure of the conjugate in circulation. The specific binding of the PS–insulin conjugates to ATIII in vivo was further substantiated in a sandwich-type ELISA employing an anti-insulin monoclonal antibody as capture and horseradish peroxidase (HRP)-conjugated anti-ATIII antibodies as detector (see Figure 4B). In this fashion, the intact PS–insulin–ATIII complex could be detected in the same plasma sam-

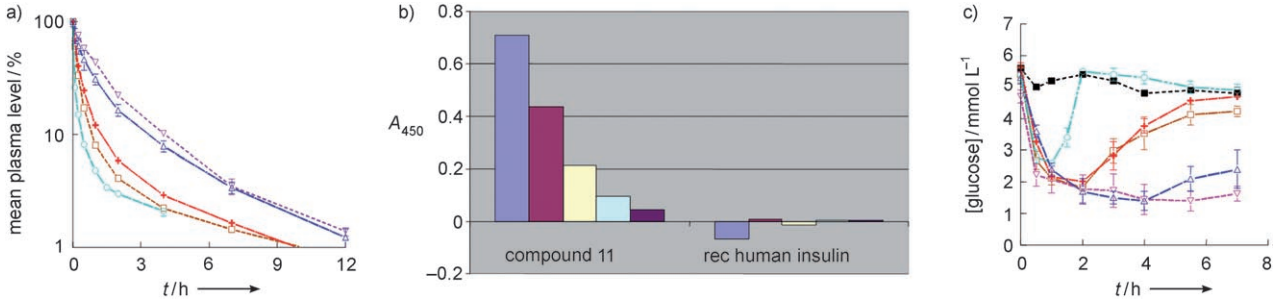


Figure 4. a) Pharmacokinetics in rat; mean plasma levels (±SEM) expressed as a percentage of the concentration measured at $t = 1$ min after i.v. administration of: ^{125}I-labeled conjugate **8** (purple ▽), **9** (brown □), **10** (red +), **11** (blue △), and recH insulin (magenta ○). b) Detection of the PS–insulin conjugate **11**: ATIII complex using an ELISA with anti-insulin/anti-rabbit ATIII antibodies; antibody dilution applied: 100× (blue), 500× (purple), 1000× (yellow), 5000× (magenta), 10000× (dark purple). c) Glucose suppression test in normoglycemic male rats; mean glucose levels (±SEM) after i.v. administration of 24 nmol kg^{-1} PS–insulin conjugate **8** (purple ▽), **9** (brown □), **10** (red +), and **11** (blue △) relative to 9 nmol kg^{-1} recH insulin (magenta ○) and control (black ■).

ples provided in the PK study, ensuring that PS–insulins are bound to ATIII in the circulation and that the prolonged half-life of ATIII-binding PSs is indeed the result of this complex formation.

The improved PK properties of insulin glycoconjugates **8**–**11** were assessed in vivo by measuring their prolonged effect on glucose suppression in non-diabetic rats in comparison with recH insulin (see Figure 4C). The increased exposure of all conjugates relative to insulin clearly gives rise to a longer duration of action. Moreover, the different duration of glucose suppression as induced by compounds **8** and **11**, compared with compounds **9** and **10**, is in line with the earlier observed differences in their ATIII-binding affinity and PK profile. Thus, after i.v. administration (24 nmol kg^{-1}), suppression of glucose levels with the "long-acting" insulin glycoconjugates **8** and **11** lasted >7 h, whereas with recH insulin (9 nmol kg^{-1}) baseline levels were restored after 2 h. Moreover, in line with their decreased affinity for ATIII, conjugates **9** and **10** showed medium duration of action, with baseline glucose levels essentially restored after 7 h. In the case of the PS–insulins no delayed onset of action was observed after s.c. administration (data not shown), and therefore the prolonged in vivo activity can be fully attributed to an increased residence time in plasma accomplished by the strong affinity for ATIII in the circulation. These results obtained in rats have been confirmed in mouse and dog models as well.

In summary, we have disclosed novel long-acting insulins based on a novel carrier technology. A combination of the available knowledge on PK/PD profiles of the synthetic ATIII-binding carrier PSs[14,20] and insulins[2,3,30] has led to the potent insulin glycoconjugates **8**–**11** in which modification at either the PheB1 or LysB29 position is tolerated. These novel compounds display attractive pharmaceutical properties, and were unambiguously characterized as highly soluble, monodisperse, monovalent, and monomeric insulin glycoconjugates. The adjustable PK/PD profile is based on a well-defined mechanism of action involving ATIII as a plasma protein carrier. Preliminary data reveal that the above-disclosed CarboCarrier™ technology[31] can be extended to other polypeptide drugs and peptide hormones in which the characteristic PK profile of the carrier PS appears to be fully transferred to the polypeptide conjugate. These results will be reported elsewhere.

Experimental Section

Conjugation: Insulin derivative **2** or **4** (25 mg) was dissolved in a sodium phosphate buffer (0.1 M, 12 mL, pH 7.0, degassed by passing N_2 through the solution). The solution was gently stirred and degassed for another 30 min. Then PS **5**, **6**, or **7**[23] (23 mg, 2.5 equiv) was added as a solid, followed by the addition of NH_2OH (50 μL, 0.05 M). The reaction mixture was stirred under a nitrogen atmosphere at ambient temperature. After 16 h the reaction mixture was subjected to preparative HPSEC (Superdex 75). The appropriate fractions were combined and lyophilized to give insulin glycoconjugates **8**, **9**, **10**, or **11** as a white powder in a typical yield of 30–50%. Yields were determined by A_{280} measurements using the same molar extinction coefficient as that of recH insulin. In all cases purities were >95% (analytical HPSEC) and >98% (analytical anion exchange). Compound **8:** yield 16 mg (47%); ESI-MS calcd for $C_{320}H_{487}N_{67}O_{137}S_{14}$: 7913, found on Q-ToF: 2637 $[M]^{3+}$, 1978 $[M]^{4+}$, 1583 $[M]^{5+}$. Compound **9:** yield 7 mg (33%); ESI-MS calcd for $C_{322}H_{491}N_{67}O_{131}S_{12}$: 7782, found on ESI Q-ToF: 2593 $[M]^{3+}$, 1945 $[M]^{4+}$, 1556 $[M]^{5+}$. Compound **10:** yield 6 mg (30%). ESI-MS calcd for $C_{321}H_{489}N_{67}O_{134}S_{13}$: 7847, found on ESI Q-ToF: 1962 $[M]^{4+}$, 1570 $[M]^{5+}$, 1308 $[M]^{6+}$. Compound **11:** yield 15 mg (45%). ESI-MS calcd for $C_{320}H_{487}N_{67}O_{137}S_{14}$: 7913, found on Q-ToF: 2638.7 $[M]^{3+}$, 1979.2 $[M]^{4+}$, 1583.6 $[M]^{5+}$, 1319.8 $[M]^{6+}$.

Pharmacokinetics: PS conjugates **8**–**11** were labeled with ^{125}I using the lactoperoxidase method. After labeling, the conjugates were purified by anion-exchange chromatography (HiTrap Q10) and administered to male Wistar rats of 300–400 g. The rats were anaesthetized by inhalation of a mixture of O_2/N_2O/isoflurane, after which the right jugular vein was cannulated. The next day rats were treated i.v. with a dose of ~1 nmol kg^{-1} (^{125}I-labeled) conjugate or recH insulin, after which blood was sampled at several time intervals. To prevent accumulation of $^{125}I^-$ in the thyroid, rats were orally treated with 10 mg kg^{-1} potassium iodide prior to administration of compound. Blood was centrifuged, after which the plasma was siphoned off and stored at −20 °C until use. The kinetic parameters were calculated with the noncompartment model of WinNonlin. Adaptation of the above-described method by measuring the radioactivity in TCA pellet (0.1 mL) yielded PK parameters that were corrected for competing endogenous ^{125}I dehalogenation.

In vivo glucose suppression in rats: The biological activities of recH insulin and compounds **8**–**11** were tested in a rat model by measuring glucose suppression. The animals were starved just prior to administration of compound to warrant consistent glucose

levels at the time of compound administration. The compounds were i.v. administered in the tail vein after pre-warming of the rats in a heating box at 39 °C for 10 min. Blood was sampled from the tail, and the glucose levels were measured with an Accu-Chek Sensor blood glucose monitor (Roche Diagnostics). Blood samples were taken at various time intervals by removing the crusted blood, after which the glucose content was determined immediately. These animal experiments were carried out in line with the Dutch policy regarding animal experimentation (Wet op de Dierproeven), issued and maintained by The Ministry of Health, Welfare and Sports (VWS).

Determination of the insulin–PS–ATIII complex: To ensure that the insulin conjugates bind to ATIII in vivo, a sandwich ELISA employing an anti-insulin MAb as capture and a HRP-conjugated anti-ATIII antibody as detector was carried out on plasma samples from the PK experiment of compound **11**. Only intact PS–insulin–ATIII complex can be detected in this type of assay (recH insulin was used as a negative control). From the plasma sample obtained after i.v. administration of 3.5 nmol kg^{-1} compound **11** or recH insulin in rat, the binding of PS–insulin conjugate **11** and recH insulin to rat ATIII was determined. The results in Figure 3 show that PS–insulin conjugate **11** is bound to ATIII (while recH insulin is not) and that anti-rabbit ATIII antibodies were able to detect the PS–insulin conjugate **11**–ATIII complex. This finding corroborates that compound **11** is bound to ATIII in the circulation and that the prolonged half-life of ATIII-binding PSs is the result of this specific interaction.

Acknowledgements

We thank Dr. André Miltenburg (N.V. Organon, part of Schering–Plough Corporation, Oss, The Netherlands) for critical reviewing of the manuscript.

Keywords: antithrombin III · glycoconjugates · insulin · oligosaccharides · protein modifications

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Received: February 22, 2008
Revised: April 7, 2008
Published online on April 24, 2008

