

PHARMACEUTICAL COMPOSITIONS OF INSULIN DERIVATIVES

TECHNICAL FIELD

The present invention relates to novel pharmaceutical compositions of insulin derivatives, wherein the insulin derivative comprises at least one aryl boron moiety and to the use of such compositions for the treatment or prevention of medical conditions relating to diabetes.

INCORPORATION-BY-REFERENCE OF THE SEQUENCE LISTING

SEQUENCE LISTING

The present application is filed with a Sequence Listing in electronic form. The entire contents of the sequence listing are hereby incorporated by reference.

BACKGROUND

In recent years, insulin derivatives displaying glucose sensitive insulin activity based on inclusion of one or more aryl boronic acid or aryl boroxol moieties have been published (WO2020/201041, WO2019/092125, WO2022/109078, WO2021/202802, WO2023/225534).

Aryl boron compounds such as aryl boronic acids may have low stability in aqueous solutions at pH near neutral value. The C-B bond can hydrolyze to give the phenyl residue and free borate, $\text{Ph-H} + \text{B(OH)}_3$, or the compound can be oxidized to give the phenolic residue + free borate, $\text{Ph-OH} + \text{B(OH)}_3$. It is thus desirable to develop stable compositions of insulin derivatives comprising one or more aryl boron moieties, such as aryl boronic acids or aryl boroxols.

SUMMARY

In the broadest aspect, the present invention relates to a liquid pharmaceutical composition of an insulin derivative, wherein the insulin derivative comprises one or more aryl boron moiety, such as aryl boronic acid or aryl boroxol moieties. It was surprisingly found that addition of methionine resulted in a more stable composition. It was also surprisingly found that addition of a sugar alcohol (polyol), such as mannitol, resulted in a more stable composition. In addition, it was found that the addition of both methionine and mannitol led to a surprisingly stable composition. In a further aspect, the composition comprises methionine and mannitol. In one aspect, the composition is stable. In one aspect, the composition is more stable than if the composition did not contain methionine. In one aspect, the composition is more stable than if the composition did not contain mannitol. In one aspect,

the composition is more stable than if the composition did not contain methionine and mannitol.

The invention may also solve further problems that will be apparent from the disclosure of the exemplary embodiments.

5 DESCRIPTION

In one aspect, a composition according to the present invention comprises

- a) an insulin derivative comprising at least one aryl boron moiety, such as an aryl boronic acid or an aryl boroxol; and
- b) methionine.

10 In one aspect, a composition according to the present invention comprises

- a) an insulin derivative comprising at least one aryl boron moiety, such as an aryl boronic acid or an aryl boroxol; and
- b) a sugar alcohol, such as mannitol or sorbitol.

In one aspect, a composition according to the present invention comprises

- 15
- a) an insulin derivative comprising at least one aryl boron moiety, such as an aryl boronic acid or an aryl boroxol;
 - b) methionine; and
 - b) a sugar alcohol, such as mannitol or sorbitol.

In one aspect, a composition according to the present invention comprises

- 20
- a) an insulin derivative comprising at least one aryl boron moiety, such as an aryl boronic acid or an aryl boroxol; and
 - b) glycerol or a sugar alcohol, such as mannitol or sorbitol, either alone or in combination.

In one aspect, a composition according to the present invention comprises

- 25
- a) an insulin derivative comprising at least one aryl boronic acid or aryl boroxol;
 - b) methionine; and
 - b) glycerol or a sugar alcohol, such as mannitol or sorbitol, either alone or in combination.

30 Insulin derivative

The term “**insulin derivative**” as used herein means a modified insulin peptide, wherein the modifications are in the form of covalent attachment of chemical moieties, also commonly referred to as sidechains or modifying groups. In one aspect, the modification is in the form of covalent attachment of at least one modifying group comprising at least one an aryl

boronic acid or aryl boroxol moiety. In one embodiment, each modifying group comprises at least two aryl boronic acid or aryl boroxol moieties. In one embodiment, each modifying group comprises at least two aryl boroxol moieties.

Examples of such insulin derivatives are disclosed in WO2020/201041, WO2019/092125,

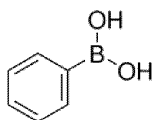
- 5 WO2022/109078, WO2021/202802, and WO2023/225534, which are incorporated herein by reference.

- 10 In some embodiments the insulin derivative may be present in the composition in its fully or partly ionised form; for example one or more carboxylic acid groups (-COOH) may be deprotonated into the carboxylate group (-COO-) and/or one or more amino groups (-NH₂) may be protonated into the -NH₃⁺ group. In some embodiments the insulin derivative is added to the composition in the form of a salt. In some embodiments the insulin derivative is added to the composition in the form of a sodium salt.

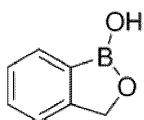
15 Aryl boronic acid and aryl boroxol

The composition of the present invention comprises an insulin derivative comprising at least one aryl boron moiety. The aryl boron moiety may be an aryl boronic acid or an aryl boroxol.

- 20 The terms "aryl boron moiety", "aryl boronic acid" and "aryl boroxol" as used herein includes substituted or unsubstituted variants. An example of an aryl boronic acid is phenylboronic acid (Chem. 1). An example of an aryl boroxol is benzoboroxol, also known as benzoxaborole (Chem. 2).

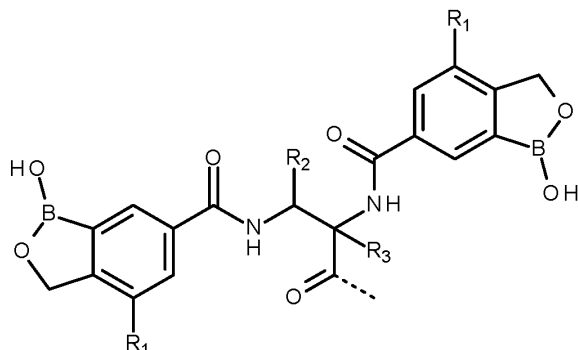


25 Chem 1. Phenylboronic acid



Chem. 2. Benzoboroxol

The aryl boron moiety may be part of a larger chemical moiety, such as Chem. 4, wherein R_1 is CF_3 , F, or H; wherein R_2 is H or CH_3 ; and wherein R_3 is H or CH_3 .



Chem. 4.

5

The moiety comprising the aryl boronic acid or the aryl boroxol are covalently attached to the insulin peptide via a chemical handle and optionally a linker. The linker may be a chemical linker or a peptide extension of the insulin peptide, or a combination thereof.

10 Insulin peptide

The term “**insulin peptide**” as used herein means a peptide which is either human insulin or a human insulin analogue. In one embodiment, the term “**insulin peptide**” as used herein means a peptide which is either human insulin or an analogue thereof with insulin activity, i.e., which activates the insulin receptor.

15

Human insulin

The term “**human insulin**” as used herein means the human insulin hormone whose structure and properties are well-known. Human insulin has two polypeptide chains, named the A-chain and the B-chain. The A-chain is a 21 amino acid peptide and the B-chain is a 30 amino acid peptide, the two chains being connected by disulphide bridges: a first bridge between the cysteine in position 7 of the A-chain and the cysteine in position 7 of the B-chain, and a second bridge between the cysteine in position 20 of the A-chain and the cysteine in position 19 of the B-chain. A third bridge is present between the cysteines in position 6 and 11 of the A-chain. The human insulin A-chain has the following sequence:

20 GIVEQCCTSICSLYQLENYCN (SEQ ID NO:1), while the B-chain has the following sequence: FVNQHLCGSHLVEALYLVCGERGFFYTPKT (SEQ ID NO:2).

25

Insulin analogue

The term “**insulin analogue**” as used herein means a modified human insulin wherein one or more amino acid residues of the insulin have been substituted by other amino acid residues and/or wherein one or more amino acid residues have been deleted from the insulin and/or wherein one or more amino acid residues have been added and/or inserted to the insulin. The term “insulin analogue” as used herein means an insulin analogue displaying insulin activity, i.e. which binds to and activates the insulin receptor.

Modifications in the insulin molecule are denoted stating the chain (A or B), the position, and the one or three letter code for the amino acid residue substituting the native amino acid residue. Herein terms like “A1”, “A2” and “A3” etc. indicates the amino acid in position 1, 2 and 3 etc., respectively, in the A chain of insulin (counted from the N-terminal end). Similarly, terms like B1, B2 and B3 etc. indicates the amino acid in position 1, 2 and 3 etc., respectively, in the B chain of insulin (counted from the N-terminal end). Using the one letter codes for amino acids, the term B29K designates that the amino acid in the B29 position is K. Using the three letter codes for amino acids, the corresponding expression is B29Lys. By “desB30” is meant an insulin analogue lacking the B30 amino acid.

In particular embodiments, an analogue “has” or “comprises” specified changes. In other particular embodiments, an analogue “consists of” the changes. When the term “consists” or “consisting” is used in relation to an analogue e.g. an analogue consists or consisting of a group of specified amino acid mutations, it should be understood that the specified amino acid mutations are the only amino acid mutations in the analogue. In contrast an analogue “comprising” a group of specified amino acid mutations may have additional mutations.

Examples of insulin analogues include:

desB30 human insulin (A-chain of SEQ ID NO:1 and B-chain of SEQ ID NO:3).

Antioxidants

Antioxidants may be used to stabilize a pharmaceutical protein in liquid formulations from oxidation. A commonly known antioxidant is ascorbic acid (Vitamin C). Addition of ascorbic acid to a composition of an insulin derivative comprising at least one aryl boronic acid or aryl boroxol led to a profound decrease in stability of the insulin derivative in the formulation.

Methionine

L-Methionine is a proteinogenic amino acid. Methionine residues in polypeptides or proteins are susceptible to oxidation, and methionine has been used as a stabilizer of formulations of polypeptides or proteins comprising methionine residues. The amount to be added should be

an amount sufficient to inhibit oxidation of the methionine residues to an acceptable level.

Generally, this can be achieved by adding methionine such that the ratio of methionine added to methionine residues ranges from about 1:1 to about 1000:1, such as 10:1 to about 100:1. Human insulin does not comprise any methionine residues, and methionine is thus not a common excipient in insulin formulations, although methionine has been reported to have antioxidant properties.

It was surprisingly found that addition of methionine to a composition of an insulin derivative comprising at least one aryl boronic acid or aryl boroxol led to an increase in stability of the insulin derivative in the formulation.

The term "methionine" as used herein includes L-methionine, D-methionine and the racemic form of methionine.

Tonicity agents

A tonicity agent has the effect of maintaining the proper osmotic pressure when a solution of the insulin derivative is being injected into the body. Commonly used tonicity agents include a salt (e.g. sodium chloride), a sugar (such as mono-, di-, or polysaccharides, or water-soluble glucans, including for example fructose, glucose, mannose, lactose, sucrose, trehalose, or dextran), a sugar alcohol (such as mannitol, sorbitol, inositol, galactitol, dulcitol, xylitol, arabitol), an amino acid (e.g. L-glycine, L-histidine, arginine, lysine, isoleucine, aspartic acid, tryptophan, threonine), an alditol (e.g. glycerol (glycerine), 1,2-propanediol (propyleneglycol), 1,3-propanediol, 1,3-butanediol), polyethyleneglycol (e.g. PEG400), or mixtures thereof. Tonicity agents are also commonly referred to as isotonic agents. The use of a tonicity agent in pharmaceutical compositions is well-known to the skilled person. For convenience reference is made to Remington: The Science and Practice of Pharmacy, 19th edition, 1995.

Glycerol and sugar alcohols as stabilizer

It was surprisingly found that addition of glycerol, which is commonly used as a tonicity agent, to a composition of an insulin derivative comprising at least one aryl boronic acid or aryl boroxol led to an increase in stability of the insulin derivative in the formulation. It was also surprisingly found that addition of the sugar alcohol mannitol in a much lower amount than when used as a tonicity agent, to a composition of an insulin derivative comprising at least one aryl boronic acid or aryl boroxol led to an increase in stability of the insulin derivative in the formulation. A similar effect was seen for the sugar alcohol sorbitol. In comparison, the addition of the sugars sucrose and trehalose had no effect on the stability of

the insulin derivative in the formulation. It is thus highly surprising that mannitol, sorbitol and/or glycerol acts as stabilizers of compositions of an insulin derivative comprising at least one aryl boronic acid or aryl boroxol.

5 The term "mannitol" as used herein includes D-mannitol, L-mannitol and the racemic form of mannitol.

Zinc

Zinc is regularly used in therapeutic insulin preparations to promote hexamer formation as a precaution against degradation during storage. The hexamer promotion is driven by
10 interactions between Zn^{2+} and insulin HisB10 residues. Zn^{2+} is usually added in form of a zinc salt, such as zinc chloride or zinc acetate.

The tested compositions comprising the insulin derivative of Example 1 were more stable in the presence of zinc than without zinc. It was surprisingly found that the tested compositions comprising the insulin derivative of Example 2 was more stable without zinc than in the
15 presence of zinc.

Preservatives

In a further embodiment of the invention the formulation further comprises a pharmaceutically acceptable preservative. The preservatives can be selected from preservatives suitable for
20 use in pharmaceutical preparations. In a further embodiment of the invention the preservative is selected from a phenolic preservative, such as phenol and m-cresol, or mixtures thereof. In a further embodiment of the invention the preservative is selected from the group consisting of phenol and m-cresol, or mixtures thereof. The use of a preservative in pharmaceutical compositions is well-known to the skilled person. For convenience reference is made to
25 Remington: The Science and Practice of Pharmacy, 19th edition, 1995.

Additional excipients

The composition of the present invention may comprise additional excipients. The term "excipient" broadly refers to any component other than the active therapeutic ingredient(s).
30 The excipient may be an inert substance, an inactive substance, and/or a not medicinally active substance. The excipient may serve various purposes, e.g. as a carrier, vehicle, diluent, and/or to improve administration, and/or absorption of the active substance. Non-limiting examples of excipients are: solvents, diluents, buffers, preservatives, tonicity regulating agents, chelating agents, and stabilisers. The formulation of pharmaceutically

active ingredients with various excipients is known in the art, see e.g. Remington: The Science and Practice of Pharmacy (e.g. 21st edition (2005), and any later editions).

A composition of the invention is in the form of a liquid formulation, i.e. aqueous formulation comprising water. A liquid formulation may be a solution, or a suspension. A composition of

- 5 the invention may be for parenteral administration, e.g. performed by subcutaneous, intramuscular, intraperitoneal, or intravenous injection.

List of embodiments

The invention is further described by the following non-limiting embodiments:

- 10 1. A liquid pharmaceutical composition comprising
- a) an insulin derivative comprising at least one aryl boron moiety; and
 - b) methionine.
2. A liquid pharmaceutical composition comprising
- a) an insulin derivative comprising at least one aryl boronic acid or aryl boroxol;
 - 15 and
 - b) methionine.
3. The liquid pharmaceutical composition according to any one of embodiments 1 to 2, wherein the concentration of methionine is within the range of 5 mM to 300 mM.
4. The liquid pharmaceutical composition according to any one of embodiments 1 to 2,
- 20 wherein the concentration of methionine is within the range of 5 mM to 60 mM.
5. The liquid pharmaceutical composition according to any one of embodiments 1 to 2, wherein the concentration of methionine is within the range of 5 mM to 45 mM.
6. The liquid pharmaceutical composition according to any one of embodiments 1 to 2, wherein the concentration of methionine is within the range of 5 mM to 30 mM.
- 25 7. The liquid pharmaceutical composition according to any one of embodiments 1 to 2, wherein the concentration of methionine is within the range of 7 mM to 20 mM.
8. The liquid pharmaceutical composition according to any one of embodiments 1 to 2, wherein the concentration of methionine is 15 mM.
9. The liquid pharmaceutical composition according to any one of embodiments 1 to 2,
- 30 wherein the concentration of methionine is 16 mM.
10. The liquid pharmaceutical composition according to any one of embodiments 1 to 2, wherein the concentration of methionine is 30 mM.
11. The liquid pharmaceutical composition according to any one of embodiments 1 to 2, wherein the concentration of methionine is 45 mM.

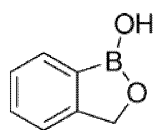
12. The liquid pharmaceutical composition according to any one of the preceding embodiments, further comprising one or more sugar alcohols.
13. The liquid pharmaceutical composition according to any one of the preceding embodiments, further comprising a sugar alcohol.
- 5 14. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the total concentration of sugar alcohol in the composition is within the range of 1 mM to 300 mM.
- 15 15. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the total concentration of sugar alcohol in the composition is within the range of 2 mM to 300 mM.
- 10 16. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the total concentration of sugar alcohol in the composition is within the range of 5 mM to 300 mM.
- 15 17. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the total concentration of sugar alcohol in the composition is within the range of 10 mM to 300 mM.
18. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the total concentration of sugar alcohol in the composition is within the range of 20 mM to 40 mM.
- 20 19. The liquid pharmaceutical composition according to any one of the preceding embodiments, further comprising mannitol.
20. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of mannitol in the composition is within the range of 10 mM to 300 mM.
- 25 21. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of mannitol in the composition is within the range of 20 mM to 40 mM.
22. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of mannitol in the composition is within the range of 40 mM to 80 mM.
- 30 23. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of mannitol in the composition is 20 mM.
24. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of mannitol in the composition is 30 mM.

25. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of mannitol in the composition is 40 mM.
26. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of mannitol in the composition is 80 mM.
- 5 27. The liquid pharmaceutical composition according to any one of the preceding embodiments, further comprising a sugar alcohol or glycerol, either alone or in combination.
28. The liquid pharmaceutical composition according to any one of the preceding embodiments, further comprising glycerol and a sugar alcohol.
- 10 29. The liquid pharmaceutical composition according to any one of the preceding embodiments, further comprising mannitol, sorbitol or glycerol, either alone or in combination.
30. The liquid pharmaceutical composition according to any one of the preceding embodiments, further comprising mannitol and glycerol.
- 15 31. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of glycerol in the composition is within the range of 10 mM to 600 mM.
32. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of glycerol in the composition is within the range of 10 mM to 300 mM.
- 20 33. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of glycerol in the composition is within the range of 100 mM to 300 mM.
34. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of glycerol in the composition is within the range of 100 mM to 230 mM.
- 25 35. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of glycerol in the composition is within the range of 150 mM to 230 mM.
- 30 36. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of glycerol in the composition is within the range of 152 mM to 217 mM.
37. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of glycerol in the composition is within the range of 185 mM to 217 mM.
- 35

38. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of glycerol in the composition is 152 mM.
39. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of glycerol in the composition is 165 mM.
- 5 40. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of glycerol in the composition is 174 mM.
41. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of glycerol in the composition is 185 mM.
42. The liquid pharmaceutical composition according to any one of the preceding
10 embodiments, wherein the concentration of glycerol in the composition is 195 mM.
43. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of glycerol in the composition is 217 mM.
44. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the pH is within the range of 5.0 to 8.0.
- 15 45. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the pH is within the range of 6.4 to 7.4.
46. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the pH is 6.6.
- 20 47. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of methionine is within the range of 5 mM to 45 mM; wherein the concentration of mannitol in the composition is within the range of 40 mM to 80 mM; and wherein the concentration of glycerol in the composition is within the range of 152 mM to 217 mM.
- 25 48. The liquid pharmaceutical composition according to any one of the preceding embodiments, further comprising a phenolic preservative or a mixture of phenolic preservatives.
49. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the phenolic preservative is phenol.
- 30 50. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the phenolic preservative is phenol in a concentration of 60 mM.
51. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the phenolic preservative is m-cresol.

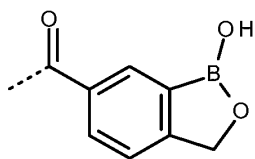
52. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the phenolic preservative is m-cresol in a concentration of 32
mM.
53. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the mixture of phenolic preservatives is phenol and m-cresol.
54. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the mixture of phenolic preservatives is phenol and m-cresol,
and wherein the concentration of phenol is in the range of 10 to 60 mM and the
concentration of metacresol is in the range of 10 to 40 mM.
55. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the mixture of phenolic preservatives is phenol and m-cresol,
and wherein the concentration of phenol is in the range of 16 to 32 mM and the
concentration of metacresol is in the range of 16 to 32 mM.
56. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the mixture of phenolic preservatives is phenol and m-cresol
and wherein the concentration of phenol is 16 mM and the concentration of
metacresol is 16 mM.
57. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the mixture of phenolic preservatives is phenol and m-cresol
and wherein the concentration of phenol is 21 mM and the concentration of
metacresol is 21 mM.
58. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the mixture of phenolic preservatives is phenol and m-cresol
and wherein the concentration of phenol is 25 mM and the concentration of
metacresol is 25 mM.
59. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the mixture of phenolic preservatives is phenol and m-cresol
and wherein the concentration of phenol is 16 mM and the concentration of
metacresol is 32 mM.
60. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the mixture of phenolic preservatives is phenol and m-cresol
and wherein the concentration of phenol is 60 mM and the concentration of
metacresol is 16 mM.
61. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the insulin derivative comprises at least 4 aryl boron moieties.

62. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises from 4 to 20 aryl boron moieties.
63. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises from 4 to 16 aryl boron moieties.
64. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises from 4 to 12 aryl boron moieties.
65. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises from 4 to 8 aryl boron moieties.
66. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises from 4 to 6 aryl boron moieties.
67. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the aryl boronic acid or aryl boroxol is an aryl boronic acid.
68. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the aryl boronic acid or aryl boroxol is an aryl boroxol.
69. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises one or more modifying groups each comprising two aryl boroxols.
70. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises from 1 to 6 modifying groups each comprising two aryl boroxols.
71. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises at least one of



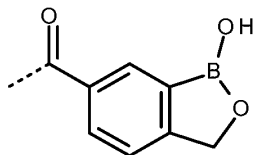
, which is optionally substituted.

72. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises at least one of



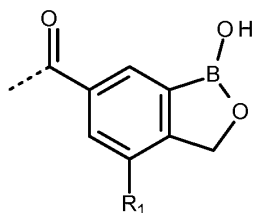
, which is optionally substituted.

73. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises at least one of



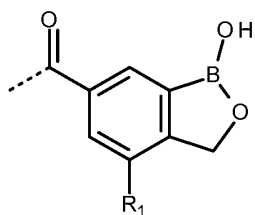
5 or more substituents selected from F, Cl, CHF₂, and CF₃.

74. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises at least one of



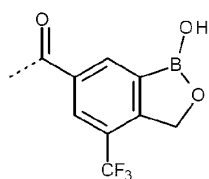
, wherein R₁ is H, F, Cl, CHF₂, or CF₃.

10 75. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises at least one of

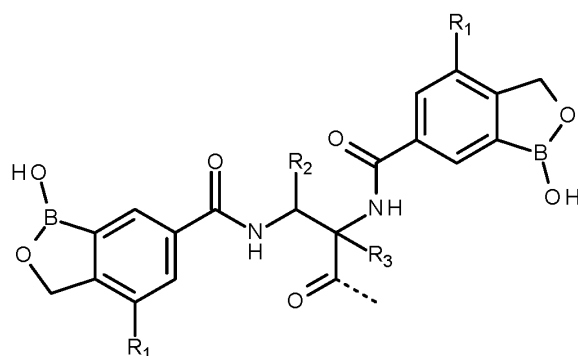


, wherein R₁ is H or CF₃.

15 76. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises at least one of



77. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises at least one of

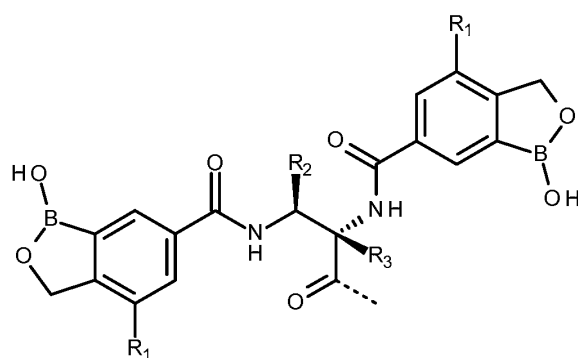


5

wherein R_1 is H, F, Cl, CHF_2 , or CF_3 ; wherein R_2 is H or CH_3 ; and wherein R_3 is H or CH_3 .

78. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises at least one of

10

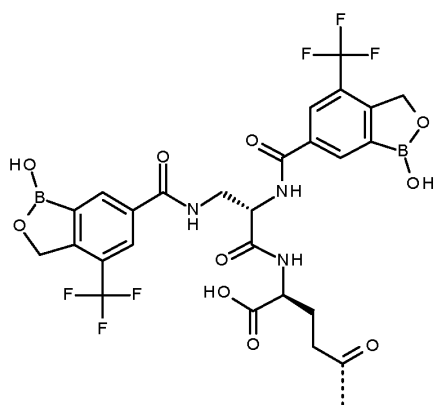


wherein R_1 is H, F, Cl, CHF_2 , or CF_3 ; wherein R_2 is H or CH_3 ; and wherein R_3 is H or CH_3 .

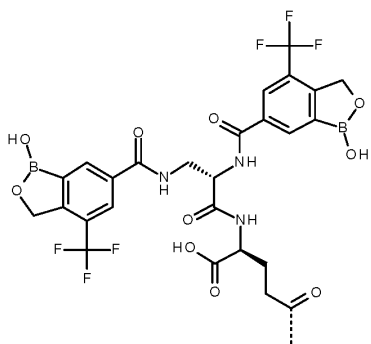
79. The liquid pharmaceutical composition according to any one of embodiments 77 to 78, wherein R_1 is H, or CF_3 .

15

80. The liquid pharmaceutical composition according to any one of embodiments 77 to 78, wherein R_2 is H; and wherein R_3 is H.
81. The liquid pharmaceutical composition according to any one of embodiments 77 to 78, wherein R_2 is H; and wherein R_3 is CH_3 .
- 5 82. The liquid pharmaceutical composition according to any one of embodiments 77 to 78, wherein R_2 is CH_3 ; and wherein R_3 is H.
83. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises at least one of

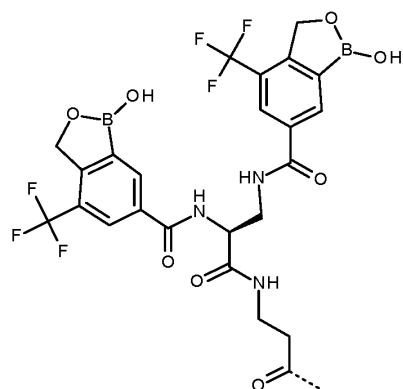


- 10 84. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises at least one of

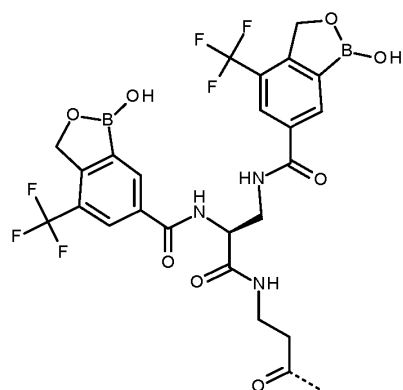


covalently attached to an amino acid residue in the insulin backbone and/or the peptide extended insulin backbone.

- 15 85. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises at least one of

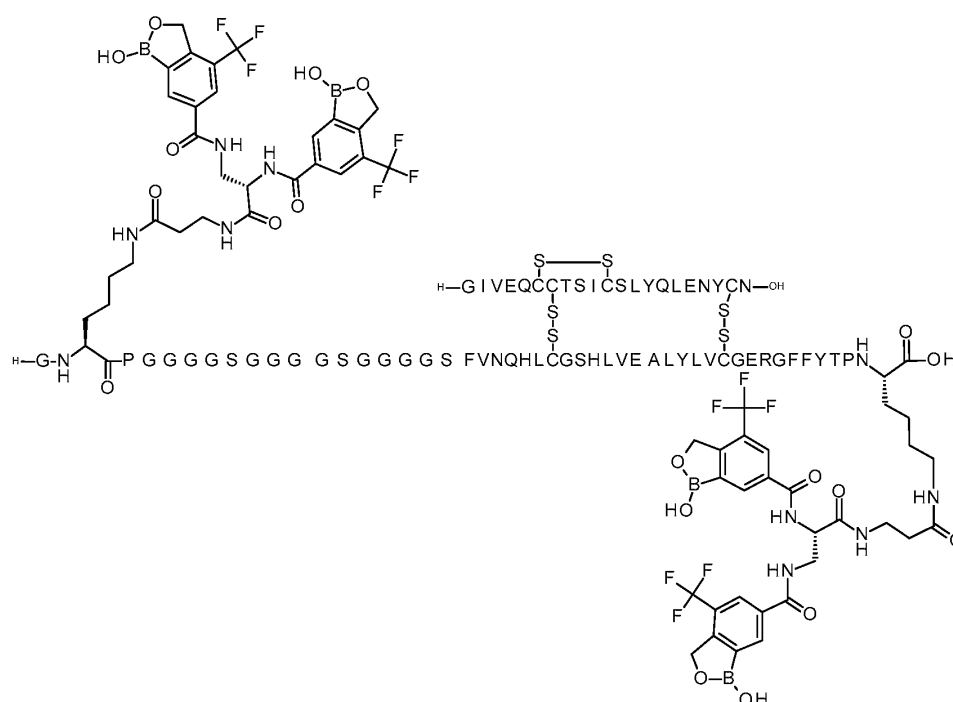


86. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprises at least one of



5 covalently attached to an amino acid residue in the insulin backbone and/or the peptide extended insulin backbone.

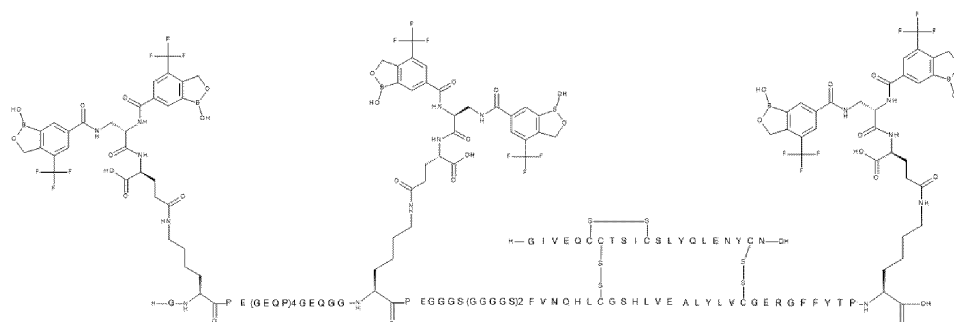
87. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative is



(the insulin derivative of Example 1), or a pharmaceutically acceptable salt thereof.

88. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative is the insulin derivative of Example 1.

5 89. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative is



(the insulin derivative of Example 2), or a pharmaceutically acceptable salt thereof.

90. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative is the insulin derivative of Example 2.

10

91. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the composition comprises:

the insulin derivative of Example 2,

glycerol,
mannitol, and
methionine.

5 92. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition comprises:

the insulin derivative of Example 2,
phenol,
metacresol,
glycerol,
10 mannitol, and
methionine.

93. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition essentially consists of:

15 the insulin derivative of Example 2,
phenol,
metacresol,
glycerol,
mannitol, and
methionine.

20 94. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition comprises:

0.6 to 4.8 mM of the insulin derivative of Example 2,
15 to 45 mM methionine,
140 to 250 mM glycerol, and
25 20 to 80 mM mannitol.

95. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition comprises 30 to 45 mM methionine.

96. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition comprises 40 to 80 mM mannitol.

30 97. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition comprises 152 to 217 mM glycerol.

98. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition is essentially free of zinc.

35 99. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition comprises:

0.6 to 4.8 mM of the insulin derivative of Example 2,
30 mM methionine,
152 mM glycerol, and
40 mM mannitol.

5 100. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition comprises:

0.6 to 4.8 mM of the insulin derivative of Example 2,
30 mM methionine,
152 mM glycerol,
10 40 mM mannitol,
25 mM phenol,
25 mM metacresol,
and wherein the pH is 6.6.

15 101. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition comprises:

0.6 mM of the insulin derivative of Example 2,
30 mM methionine,
152 mM glycerol,
40 mM mannitol,
20 25 mM phenol,
25 mM metacresol,
and wherein the pH is 6.6.

25 102. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition comprises:

1.2 mM of the insulin derivative of Example 2,
30 mM methionine,
152 mM glycerol,
40 mM mannitol,
25 mM phenol,
30 25 mM metacresol,
and wherein the pH is 6.6.

35 103. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition comprises:

2.4 mM of the insulin derivative of Example 2,
30 mM methionine,

152 mM glycerol,
40 mM mannitol,
25 mM phenol,
25 mM metacresol,
5 and wherein the pH is 6.6.

104. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition comprises:
3.6 mM of the insulin derivative of Example 2,
30 mM methionine,
10 152 mM glycerol,
40 mM mannitol,
25 mM phenol,
25 mM metacresol,
and wherein the pH is 6.6.

15 105. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition comprises:
4.8 mM of the insulin derivative of Example 2,
30 mM methionine,
152 mM glycerol,
20 40 mM mannitol,
25 mM phenol,
25 mM metacresol,
and wherein the pH is 6.6.

25 106. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition essentially consists of:
0.6 to 4.8 mM of the insulin derivative of Example 2,
30 mM methionine,
152 mM glycerol,
40 mM mannitol,
30 25 mM phenol,
25 mM metacresol,
and wherein the pH is 6.6.

107. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition essentially consists of:
35 0.6 mM of the insulin derivative of Example 2,

30 mM methionine,
152 mM glycerol,
40 mM mannitol,
25 mM phenol,
5 25 mM metacresol,
and wherein the pH is 6.6.

108. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition essentially consists of:
1.2 mM of the insulin derivative of Example 2,
10 30 mM methionine,
152 mM glycerol,
40 mM mannitol,
25 mM phenol,
25 mM metacresol,
15 and wherein the pH is 6.6.

109. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition essentially consists of:
2.4 mM of the insulin derivative of Example 2,
30 mM methionine,
20 152 mM glycerol,
40 mM mannitol,
25 mM phenol,
25 mM metacresol,
and wherein the pH is 6.6.

25 110. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition essentially consists of:
3.6 mM of the insulin derivative of Example 2,
30 mM methionine,
152 mM glycerol,
30 40 mM mannitol,
25 mM phenol,
25 mM metacresol,
and wherein the pH is 6.6.

111. The liquid pharmaceutical composition according to any one of the preceding
35 embodiments, wherein the composition essentially consists of:

4.8 mM of the insulin derivative of Example 2,
30 mM methionine,
152 mM glycerol,
40 mM mannitol,
5 25 mM phenol,
25 mM metacresol,
and wherein the pH is 6.6.

112. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition comprises:
10 0.6 to 4.8 mM of the insulin derivative of Example 2,
16 mM phenol,
16 mM metacresol,
185 mM glycerol,
40 mM mannitol,
15 30 mM methionine,
and wherein the pH is 6.6.

113. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition essentially consists of:
0.6 to 4.8 mM of the insulin derivative of Example 2,
20 16 mM phenol,
16 mM metacresol,
185 mM glycerol,
40 mM mannitol,
30 mM methionine,
25 and wherein the pH is 6.6.

114. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition is essentially free of zinc.

115. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein the composition is essentially free of zinc ions.

30 116. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein when the insulin derivative is the insulin derivative of Example
2, then the composition is essentially free of zinc.

117. The liquid pharmaceutical composition according to any one of the preceding
embodiments, wherein when the insulin derivative is the insulin derivative of Example
35 2, then the composition is essentially free of zinc ions.

118. A liquid pharmaceutical composition according to any one of embodiments 1 to 117 for use as a medicament.
119. A liquid pharmaceutical composition according to any one of embodiments 1 to 117, for use in the prevention or treatment of diabetes, diabetes of Type 1, diabetes of Type 2, impaired glucose tolerance, hyperglycemia, and metabolic syndrome (metabolic syndrome X, insulin resistance syndrome).
120. A liquid pharmaceutical composition according to any one of embodiments 1 to 117, for use in the prevention or treatment of diabetes.
121. A liquid pharmaceutical composition according to any one of embodiments 1 to 117, for use in the prevention or treatment of type 1 diabetes and type 2 diabetes.
122. Use of the liquid pharmaceutical composition according to anyone of embodiments 1 to 117, for the manufacture of a medicament for the treatment or prevention of diabetes, diabetes of Type 1, diabetes of Type 2, impaired glucose tolerance, hyperglycemia, and metabolic syndrome (metabolic syndrome X, insulin resistance syndrome).
123. A method for the treatment or prevention of diabetes, diabetes of Type 1, diabetes of Type 2, impaired glucose tolerance, hyperglycemia, and metabolic syndrome (metabolic syndrome X, insulin resistance syndrome), which method comprises administration to a subject in need thereof a therapeutically effective amount of the liquid pharmaceutical composition according to any one of embodiments 1 to 117.
124. A method for the treatment of diabetes in a subject comprising administering to said subject a liquid pharmaceutical composition according to any one of embodiments 1 to 117.
- The invention is further defined by this second list of embodiments:
1. A liquid pharmaceutical composition comprising
 - a) an insulin derivative comprising at least one aryl boronic acid or aryl boroxol; and
 - b) methionine.
 2. The liquid pharmaceutical composition according to embodiment 1, wherein the concentration of methionine is within the range of 5 mM to 300 mM.
 3. The liquid pharmaceutical composition according to any one of the preceding embodiments, further comprising glycerol or a sugar alcohol, such as mannitol or sorbitol, either alone or in combination.

4. The liquid pharmaceutical composition according to any one of the preceding embodiments, further comprising mannitol.
5. The liquid pharmaceutical composition according to any one of the preceding embodiments, further comprising mannitol and glycerol.
- 5 6. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of mannitol in the composition is within the range of 10 mM to 300 mM.
7. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of glycerol in the composition is within the range of 10 mM to 600 mM.
- 10 8. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the pH is within the range of 6.4 to 7.4.
9. The liquid pharmaceutical composition according to any one of the preceding embodiments, further comprising a phenolic preservative or a mixture of phenolic preservatives.
- 15 10. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the aryl boronic acid or aryl boroxol is an aryl boroxol.
11. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the insulin derivative comprising at least one aryl boronic acid or aryl boroxol is the insulin derivative of Example 1 or the insulin derivative of Example 2.
- 20 12. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the composition comprises:
the insulin derivative of Example 2,
25 phenol,
metacresol,
glycerol,
mannitol, and
methionine.
- 30 13. The liquid pharmaceutical composition according to any one of the preceding embodiments, wherein the composition comprises:
0.6 to 4.8 mM of the insulin derivative of Example 2,
16 mM phenol,
16 mM metacresol,
35 185 mM glycerol,

40 mM mannitol,
30 mM methionine,
and wherein the pH is 6.6.

- 5
14. A liquid pharmaceutical composition according to any one of the preceding
embodiments, for use as a medicament.
15. A liquid pharmaceutical composition according to any one of the preceding
embodiments, for use in the prevention or treatment of diabetes, diabetes of Type 1,
diabetes of Type 2, impaired glucose tolerance, hyperglycemia, and metabolic
syndrome (metabolic syndrome X, insulin resistance syndrome).

10

EXAMPLES

Materials and Methods

The mannitol used in the following examples is D-mannitol.

- 15
- The sorbitol used in the following examples is D-sorbitol.

The methionine used in the following examples is L-methionine.

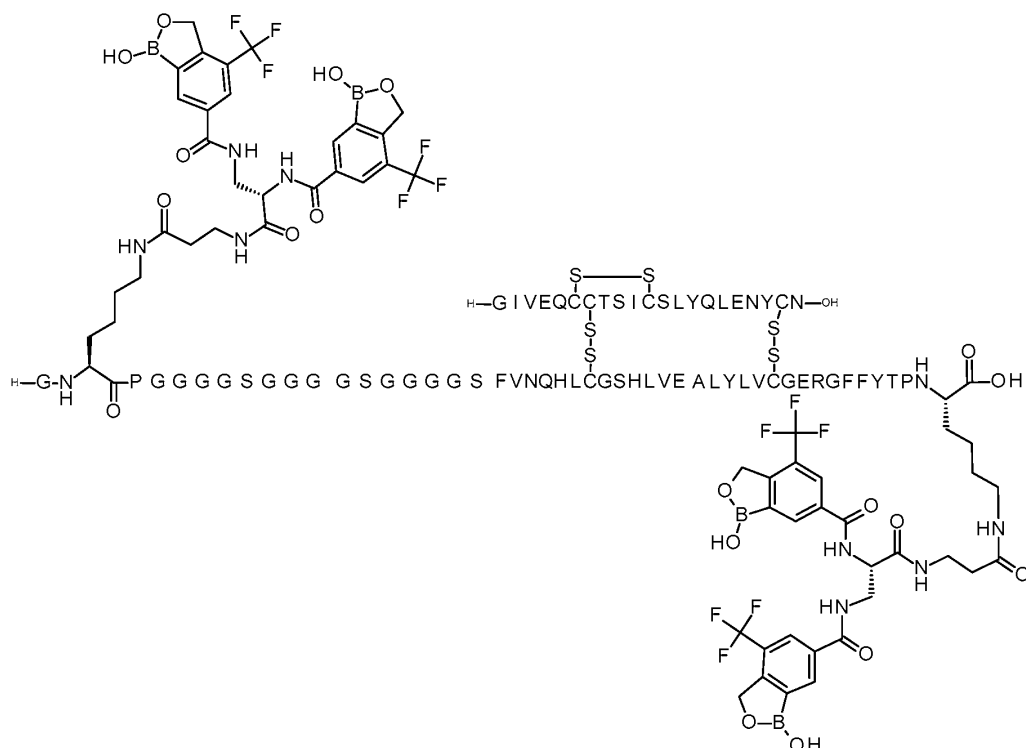
The ascorbate (sodium ascorbate) used in the following examples is L-ascorbate (sodium L-ascorbate).

20

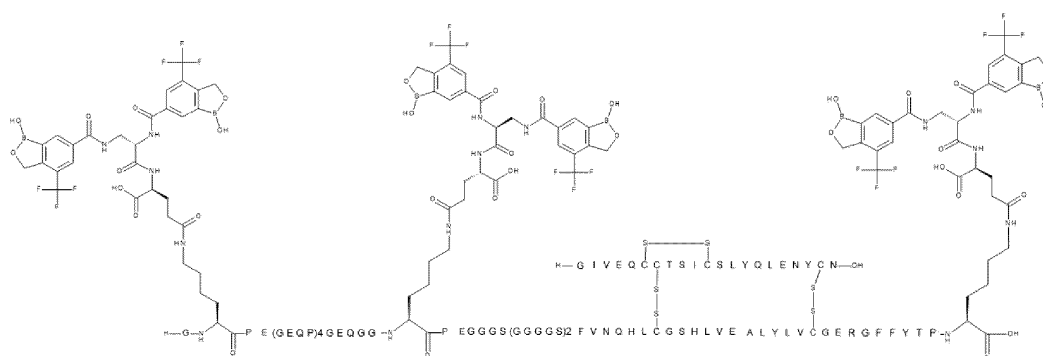
List of Abbreviations

ACN	acetonitrile
ALP	achromobactor lyticus protease
BEH	Ethylene Bridged Hybrid (Ethylene Bridged Hybrid particle technology)
25 C18	octadecanyl (HPLC column)
CV	column volume
Dap	2,3-Diaminopropionic acid
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
DIC	<i>N,N</i> -diisopropylcarbodiimide
30 DMF	<i>N,N</i> -dimethylformamide
HATU	1-((dimethylamino)(dimethyliminio)methyl)-1H-[1,2,3]triazolo-[4,5-b]pyridine 3-oxide hexafluorophosphate
HCl	hydrochloric acid
HPLC	high permance liquid chromatography

	hrs	hours
	LCMS	liquid chromatography mass spectrometry
	min	minutes
	MeCN	acetonitrile
5	mm	millimeter
	mM	millimolar
	NaCl	sodium chloride
	NaOH	sodium hydroxide
	NBS	<i>N</i> -bromosuccinimide
10	NHS	N-hydroxysuccinimide
	NMR	nuclear magnetic resonance
	q.s.	quantum satis
	RP-HPLC	reverse-phase high performance liquid chromatography
	tBu	tert-butyl
15	TFA	trifluoroacetic acid
	UPLC	ultra high performance liquid chromatography
	UV	ultraviolet
	Å	Angstrom

Example 1: Preparation of the insulin derivative of Example 1

The insulin derivative of Example 1 is disclosed in WO2020/201041 (Example 280) and was
 5 prepared as described in WO2020/201041 (the insulin A-chain has the sequence of SEQ ID
 NO:1 and the extended B-chain has the sequence of SEQ ID NO:4).

Example 2: Preparation of the insulin derivative of Example 2

Expression of insulin variant in yeast and transformation with ALP etc

The insulin analogue GKPE-(GEQP)₄-GEQGGKPEGGGSGGGGSGGGGS-B1 desB30 human insulin (the insulin A-chain has the sequence of SEQ ID NO:1 and the extended B-chain has the sequence of SEQ ID NO:5) was expressed in yeast using well-known

5 techniques e.g. as disclosed in WO2017/032798. More specifically, the insulin analogue was expressed as a single-chain precursor, which were isolated by ion-exchange capture, and cleaved to the 2-chain insulin analogue by treatment with ALP as described below.

GKPE-(GEQP)₄-GEQG-GKPEGGGSGGGGSGGGGS-B1 desB30 human insulin means desB30 human insulin extended from B1 with GKPE-GEQPGEQPGEQPGEQP-GEQG-

10 GKPEGGGSGGGGSGGGGS (SEQ ID NO:6) (C-terminal S connected to B1F).

Capture of the precursor on SP Sepharose BB:

The yeast supernatant was loaded with a flow of 10-20 CV/h onto a column packed with SP Sepharose BB. A wash with 0.1 M citric acid pH 3.5 and a wash with 40% EtOH were

15 performed. The analogue was eluted with 0.2 M sodium acetate pH 5,5 / 35 % EtOH.

ALP digestion:

The solution of single-chain precursor was adjusted to pH 9 and ALP enzyme was added 1:100 (w/w). The reaction was followed on UPLC. ALP cleavage pool was adjusted to pH 2,5

20 and diluted 2-fold in order to be prepared for RP-HPLC purification.

RP-HPLC Purification:

Purification was performed by RP-HPLC C18 as below:

Column: 15um C18 50x250mm 200Å

25 Buffers:

A: 0.2% formic acid, 5 % EtOH,

B: 0.2% formic acid, 50 % EtOH

The gradient: 20-55 % B-buffer.

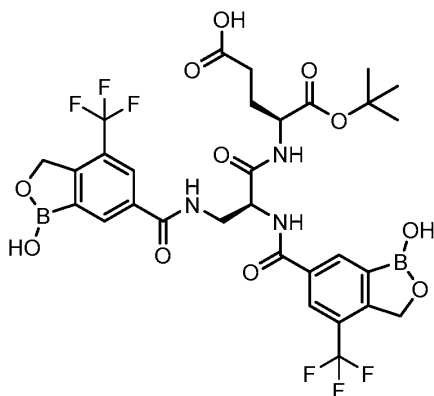
Gradient: 20 CV

30 Flow 20 CV/h

Load g ~ 5 g/l resin

Fractions were analysed by UPLC, pooled and freeze dried.

Preparation of (S)-4-((S)-2,3-Bis(1-hydroxy-4-(trifluoromethyl)-1,3-dihydrobenzo[c][1,2]oxaborole-6-carboxamido)propanamido)-5-(tert-butoxy)-5-oxopentanoic acid (building block 1)



- 5 NBS (34.0 g, 191 mmol) was added to a solution of 3-trifluoromethyl-4-methylbenzoic acid (39.0 g, 191 mmol) in concentrated sulfuric acid (400 mL) and the reaction mixture was stirred at room temperature for 16 hrs. The reaction mixture was then poured into ice-water (2 L). Resulting precipitate was filtered off, washed with water (500 mL) and dissolved in ethyl acetate (400 mL); dried over anhydrous sodium sulfate, filtered and evaporated to
- 10 provide 3-bromo-4-methyl-5-trifluoromethylbenzoic acid as white solid. Yield: 53.4 g (98%). ¹H NMR spectrum (300 MHz, DMSO-d₆, δH): 13.71 (bs, 1 H); 8.35 (d, J=0.4 Hz, 1 H); 8.15 (d, J=0.9 Hz, 1 H); 2.56 (s, 3 H).

- Concentrated sulfuric acid (24 mL) was added to a solution 3-bromo-4-methyl-5-
- 15 trifluoromethylbenzoic acid (35.0 g, 124 mmol) in methanol (500 mL) and the reaction mixture was allowed to stir under reflux for 4 hrs and at room temperature for 16 hrs. The reaction mixture was then evaporated under reduced pressure, dissolved in diethyl ether (250 mL), washed with water (2 x 100 mL) and mixture of saturated solution of potassium carbonate (100 mL) and brine (100 mL). Organic layer was separated, dried over anhydrous
- 20 sodium sulfate, filtered and evaporated to provide methyl 3-bromo-4-methyl-5-trifluoromethylbenzoate as white solid. Yield: 35.3 g (96%). ¹H NMR spectrum (300 MHz, DMSO-d₆, δH): 8.36 (d, J=1.1 Hz, 1 H); 8.13 (d, J=1.1 Hz, 1 H); 3.90 (s, 3 H); 2.55 (d, J=1.3 Hz, 3 H).

- 25 A suspension of NBS (31.7 g, 178 mmol) and methyl 3-bromo-4-methyl-5-trifluoromethylbenzoate (35.3 g, 119 mmol) in water (300 mL) was stirred for 6 hrs under 100 W light bulb at 80 °C. Reaction mixture was extracted with diethyl ether (2 x 200 mL).

Organic layers were washed with brine (150 mL). Organic layer was separated, dried over anhydrous sodium sulfate, filtered and evaporated to provide methyl 3-bromo-4-bromomethyl-5-trifluoromethylbenzoate as yellow solid. Yield: 44.0 g (98%). ¹H NMR spectrum (300 MHz, CDCl₃, δH): 8.47 (d, J=1.5 Hz, 1 H); 8.31 (d, J=1.3 Hz, 1 H); 4.75 (s, 2 H); 3.98 (s, 3 H).

Solution of methyl 3-bromo-4-bromomethyl-5-trifluoromethylbenzoate (44.0 g, 117 mmol) and potassium acetate (22.9 g, 234 mmol) in MeCN (0.5 L) was stirred at 75 °C overnight. The suspension was filtered through filtering paper and evaporated. The crude product was dissolved in dichloromethane and filtered again. Evaporation provided methyl 4-(acetoxymethyl)-3-bromo-5-(trifluoromethyl)benzoate as white solid. Yield: 37.9 g (91%). ¹H NMR spectrum (300 MHz, CDCl₃, δH): 8.49 (d, J=1.3 Hz, 1 H); 8.34 (d, J=1.3 Hz, 1 H); 5.37 (s, 2 H); 3.99 (s, 3 H); 2.11 (s, 3 H).

A solution of methyl 4-(acetoxymethyl)-3-bromo-5-(trifluoromethyl)benzoate (37.9 g, 107 mmol), bis(pinacolato)diboron (29.8 g, 117 mmol), potassium acetate (31.4 g, 294 mmol) and [1,1-bis(diphenylphosphino)ferrocene]dichloropalladium(II) (1.57 g, 1.92 mmol) in dry tetrahydrofuran (500 mL) was allowed to stir at 75 °C under argon atmosphere for 13 days. Then the reaction mixture was cooled to room temperature, filtered and evaporated. The crude product was filtered through silica gel column (Silicagel, 0.063-0.200 mm; eluent: cyclohexane/ethyl acetate 8:1) to provide methyl 4-(acetoxymethyl)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-5-(trifluoromethyl)benzoate. Yield: 31.1 g (72%). ¹H NMR spectrum (300 MHz, CDCl₃, δH): 8.65 (s, 1 H); 8.43 (s, 1 H); 5.48 (s, 2 H); 3.97 (s, 3 H); 2.05 (s, 3 H); 1.36 (s, 12 H).

A solution of methyl 4-(acetoxymethyl)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-5-(trifluoromethyl)benzoate (31.0 g, 77.1 mmol) and sodium hydroxide (15.4 g, 386 mmol) in water (300 mL) was stirred at room temperature for 3 hrs. Then solution of hydrochloric acid (35 mL) in water (100 mL) was added to lower the pH to 1. The reaction mixture was stirred overnight. Precipitate was filtered off and dried to provide 1-hydroxy-4-(trifluoromethyl)-1,3-dihydrobenzo[c][1,2]oxaborole-6-carboxylic acid as white solid. Yield: 16.6 g (86%). ¹H NMR spectrum (300 MHz, DMSO-d₆, δH): 13.47 (bs, 1 H); 9.66 (s, 1 H); 8.62 (s, 1 H); 8.24 (s, 1 H); 5.22 (s, 2 H).

A solution of pentafluorophenol (7.48 g, 40.7 mmol), 1-hydroxy-4-(trifluoromethyl)-1,3-dihydrobenzo[c][1,2]oxaborole-6-carboxylic acid (10.0 mg, 40.7 mmol) and N,N'-dicyclohexylcarbodiimide (DCC, 8.37 mg, 40.7 mmol) in MeCN (0.5 L) was stirred at room temperature overnight. The reaction mixture was filtered, evaporated, dissolved in MeCN, re-
5 filtered and evaporated to give the pentafluorophenyl 1-hydroxy-4-(trifluoromethyl)-1,3-dihydrobenzo[c][1,2]oxaborole-6-carboxylate as white solid.

Yield: 16.7 g (100%). ¹H NMR spectrum (300 MHz, DMSO-d₆, δH): 9.79 (s, 1 H); 8.86 (s, 1 H); 8.46 (s, 1 H); 5.30 (s, 2 H).

- 10 2-Chlorotrityl chloride resin 100-200 mesh 1.5 mmol/g (3, 4.47 g, 6.71 mmol) was left to swell in dry dichloromethane (30 mL) for 30 min. A solution of (2S)-5-(tert-butoxy)-2-[(9H-fluoren-9-ylmethoxy)carbonyl]amino-5-oxopentanoic acid (Fmoc-Glu-OtBu, 1.90 g, 4.47 mmol) and N,N-diisopropylethylamine (2.96 mL, 17.0 mmol) in dry dichloromethane (30 mL) was added to resin and the mixture was shaken overnight. Resin was filtered and treated with a solution
15 of N,N-diisopropylethylamine (1.56 mL, 8.95 mmol) in methanol/dichloromethane mixture (4:1, 2 x 5 min, 2 x 40 mL). Then resin was washed with DMF (2 x 30 mL), dichloromethane (2 x 40 mL) and DMF (3 x 40 mL). Fmoc group was removed by treatment with 20% piperidine in DMF (1 x 5 min, 1 x 20 min, 2 x 40 mL). Resin was washed with DMF (3 x 40 mL), 2-propanol (2 x 40 mL) and dichloromethane (3 x 40 mL). Solution of (S)-2,3-bis(((9H-fluoren-9-yl)methoxy)carbonyl)amino)propanoic acid (Fmoc-Dap(Fmoc)-OH, 3.68 g, 6.71
20 mmol), 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU, 2.55 g, 6.71 mmol) and 2,4,6-trimethylpyridine (1.60 mL, 12.1 mmol) in DMF (40 mL) was added to resin and mixture was shaken for 2 hrs. Resin was filtered and washed with DMF (2 x 40 mL), dichloromethane (2 x 40 mL) and DMF (2 x 40
25 mL). Fmoc groups were removed by treatment with 20% piperidine in DMF (1 x 5 min, 1 x 30 min, 2 x 40 mL). Resin was washed with DMF (3 x 40 mL), 2-propanol (2 x 40 mL) and dichloromethane (3 x 40 mL). Solution of pentafluorophenyl 1-hydroxy-4-(trifluoromethyl)-1,3-dihydrobenzo[c][1,2]oxaborole-6-carboxylate (5.53 g, 13.4 mmol) and triethylamine (4.99 mL, 35.8 mmol) in DMF (40 mL) was added to resin and mixture was shaken overnight. Resin
30 was filtered and washed with DMF (6 x 40 mL) and dichloromethane (10 x 50 mL). The product was cleaved from resin by treatment with 2,2,2-trifluoroethanol (60 mL) for 16 hrs. Resin was filtered off and washed with dichloromethane (4 x 50 mL). Crude product (4) was dried in vacuo and extracted with ethyl acetate (2 x 70 mL) and 1 M aqueous solution of potassium hydrogen sulfate (50 mL), organic phases were dried over anhydrous sodium
35 sulfate, filtered and the solvent was evaporated. Crude product was then triturated in diethyl

ether (20 mL) to give (S)-4-((S)-2,3-bis(1-hydroxy-4-(trifluoromethyl)-1,3-dihydrobenzo[c][1,2]oxaborole-6-carboxamido)propanamido)-5-(tert-butoxy)-5-oxopentanoic acid (building block 1) as beige solid. Yield: 1.89 g (57%). ¹H NMR spectrum (300 MHz, AcOD-d₄, δH): 8.50 (s, 1 H); 8.46 (s, 1 H); 8.29 (s, 1 H); 8.26 (s, 1 H); 5.28 (d, J=2.6 Hz, 4 H); 5.20 (t, J=5.9 Hz, 1 H); 4.55 (dd, J=8.5 and 5.2 Hz, 1 H); 4.08 (dd, J=6.0 and 2.1 Hz, 2 H); 2.57-2.42 (m, 2 H); 2.34-2.16 (m, 1 H); 2.17-2.08 (m, 1 H); 1.47 (s, 9 H). LC-MS: 746.3 (M+H)⁺.

Preparation of insulin derivative

Building block 1 was activated with NHS/DIC in THF and the tBu protection group was removed by treatment with TFA. The TFA was then evaporated prior to conjugation with insulin.

GKPE-(GEQP)₄-GEQGGKPEGGGSGGGSGGGGS-B1 desB30 human insulin (536 mg, 0.057 mmol) was dissolved in 0.1 M Na₂HPO₄ (5.6 mL) and DMSO (2.4 mL) and pH was adjusted to 10.8 by 1.0 M aq. NaOH. Building block 1 (147 mg, 0.19 mmol) was NHS activated and tBu deprotected as described above, then dissolved in DMF (0.5 mL), and added dropwise over 10 mins to the given insulin solution, while pH was kept near 10.8 by dropwise addition of 0.1 M NaOH. LCMS shows formation of the desired product. The mixture was diluted with MeCN and water, and pH was adjusted to 3.8 by dropwise addition of 1 M HCl. The product was purified by reverse-phase HPLC (RP-HPLC) on C18 column using 0.1% TFA in water as buffer A and 0.1% TFA in MeCN as buffer B. The product was isolated by lyophilisation. LCMS measured 1894.1 [M + 6H - 5x water]⁶⁺, calculated 1894.1; 1623.6 [M + 7H - 5x water]⁷⁺, calculated 1623.6; 1420.8 [M + 8H - 5x water]⁸⁺, calculated 1420.8; and 1263.0 [M + 9H - 5x water]⁹⁺, calculated 1263.0, for C₄₈₀H₆₆₆B₆F₁₈N₁₂₂O₁₆₉S₆.

Example 3: Effect on stability from antioxidants methionine and sodium ascorbate

In this example the stabilizing effect of the two antioxidants methionine and sodium ascorbate were investigated. Chemical stability can be measured in terms of purity, where the content of the intact insulin derivative is determined at time zero and upon storage at a specific temperature for a specific time. The tested formulations are shown in Table 1.

Preparation process

Each formulation was prepared from the following stock solutions; 2 mM insulin derivative of Example 1, 100 mM sodium phosphate, 500 mM phenol, 160 mM metacresol, 1

M NaCl, 2.17 M glycerol, 10 mM zinc acetate, 1.34 M methionine, 1.01 M sodium ascorbate, water for injection.

The formulations were pH adjusted as necessary using HCl and NaOH and water was added to reach the final desired volumes before being sterilised by filtration through a 0.22 µm sterile filter. After filtration, the formulations were filled into HPLC vials to contain 50 µl for time zero analyses and filled into 3 ml sized drug cartridges to contain 0.5 ml of formulation for storage stability analyses.

Methods and assay experimentals

The cartridges were stored for 4 weeks at 37°C and analysed for purity which were compared to the results from analyses of the time point zero samples.

Purity (as percentage) for the insulin derivative of Example 1 was determined using UPLC equipped with a Waters column BEH C18, 2.1x150 mm, 1.7 µm, 130Å gradient eluted over an approximately 64-minute run time using 0.1% TFA in milliQ water (buffer A) and 0.09% TFA in 90% ACN (buffer B) and using 9% acetonitrile as wash buffer. The column temperature was 35°C and the injection volume was 1 µl while the flow rate was set to 0.3 ml/min. Chromatography was analysed by UV detection (215 nm) and purity was evaluated as the area of the main peak divided by the area of all peaks x 100%.

Results

The purity (as percentages) at time zero and after storage for 4 weeks at 37°C for Formulations 1 to 5 are shown in Table 1. Purity loss is also provided as the difference (in percentage points) between the purity after 4 weeks at 37 °C and time zero.

The purity loss after 4 weeks at 37°C was 8.7 percentage points for Formulation 1 having no antioxidant. Surprisingly, it was found that whereas sodium ascorbate led to a purity loss of more than 50 percentage points after 4 weeks, addition of methionine improved the stability of the insulin formulation.

30

Table 1. Effect on stability of methionine and ascorbic acid

Ingredients	Formulation				
	1	2	3	4	5
Insulin derivative of Example 1 (mM)	0.6	0.6	0.6	0.6	0.6
Zinc acetate (mM)	0.24	0.24	0.24	0.24	0.24
Methionine (mM)	-	7	20	-	-
Sodium ascorbate (mM)	-	-	-	5.0	15.1
Glycerol (mM)	195	195	195	195	195
Phenol (mM)	16	16	16	16	16
Metacresol (mM)	16	16	16	16	16
Sodium chloride (mM)	10	10	10	10	10
Sodium phosphate (mM)	7	7	7	7	7
HCl	q.s.	q.s.	q.s.	q.s.	q.s.
NaOH	q.s.	q.s.	q.s.	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml
pH	7.4	7.4	7.4	7.4	7.4
Purity (%), time zero	94.5	95.1	94.8	87.5	81.4
Purity (%), 4 weeks at 37°C	85.8	87.4	88.4	25.9	25.8
Purity loss (% points), 4 weeks at 37°C	8.7	7.7	6.4	61.6	55.7

Example 4: Effect on stability from glycerol, sugar alcohols and sugars

- 5 In this example the stabilizing effect of glycerol, sugar alcohols and sugars on formulations of the insulin derivative of Example 1 was investigated. The tested formulations are shown in Table 2.

Preparation process

- 10 Each formulation was prepared from the following stock solutions; 2 mM insulin derivative of Example 1, 100 mM sodium phosphate, 500 mM phenol, 160 mM metacresol, 1 M NaCl, 2.17 M glycerol, 10 mM zinc acetate, 200 mM sorbitol, 300 mM mannitol, 200 mM sucrose, 200 mM trehalose, water for injection.

- 15 The formulations were pH adjusted as necessary using HCl and NaOH and water was added to reach the final desired volumes before being sterilised by filtration through a 0.22 µm sterile filter. After filtration, the formulations were filled into HPLC vials to contain 50

µl for time zero analyses and filled into 3 ml sized drug cartridges to contain 0.5 ml of formulation for storage stability.

Methods and assay experimentals

5 The cartridges were stored for 1 week at 37°C and analysed for purity. In addition, the cartridges were stored for an additional 6 days at 45°C to further promote the degradation and analysed for purity.

10 Purity (as percentage) for the insulin derivative of Example 1 was determined using UPLC equipped with a Waters column BEH C18, 2.1x150 mm, 1.7 µm, 130Å gradient eluted over an approximately 70-minute run time using 0.1% TFA in milliQ water (buffer A) and 0.09% TFA in 90% ACN (buffer B) and using 9% acetonitrile as wash buffer. The column temperature was 50°C and the injection volume was 2 µl while the flow rate was set to 0.3 ml/min. Chromatography was analysed by UV detection (215 nm) and purity was evaluated
15 as the area of the main peak divided by the area of all peaks x 100%.

Results

 The purity (as percentages) at time zero and after storage for 1 week at 37°C as well as 1 week at 37°C 1 plus additional 6 days at 45°C for Formulations 6 to 15 are shown
20 in Table 2a and Table 2b. Purity loss is also provided as the difference (in percentage points) between the purity after storage and time zero.

 The purity loss after 1 week at 37°C plus additional 6 days at 45°C was 12.3 percentage points for Formulation 6 having neither glycerol, sugar alcohol nor sugar in the
25 formulation.

 Tonicity agents are commonly used in pharmaceutical formulations intended for subcutaneous injection to ensure proper osmotic pressure of the formulation. It was surprisingly found that stability was improved greatly by addition of the tonicity agent glycerol in a concentration of 174 mM (Formulation 7).

30 Even more surprisingly, stability was even better for Formulation 8 containing only 20 mM of the sugar alcohol mannitol, resulting in a purity loss of 5.0 percentage points after 1 week at 37°C plus additional 6 days at 45°C.

 Surprisingly, the stability could be improved even further by the combination of 174 mM glycerol and 20 mM mannitol (Formulation 10).

35

Table 2a. Effect on stability of mannitol, sorbitol, sucrose and trehalose

Ingredients	Formulation						
	6	7	8	10	13	14	15
Insulin derivative of Example 1 (mM)	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Zinc acetate (mM)	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Mannitol (mM)	-	-	20	20	-	-	-
Sorbitol (mM)	-	-	-	-	20	-	-
Sucrose (mM)	-	-	-	-	-	20	-
Trehalose (mM)	-	-	-	-	-	-	20
Glycerol (mM)	-	174	-	174	174	174	174
Phenol (mM)	16	16	16	16	16	16	16
Metacresol (mM)	16	16	16	16	16	16	16
Sodium chloride (mM)	10	10	10	10	10	10	10
Sodium phosphate (mM)	7	7	7	7	7	7	7
HCl	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
NaOH	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml
pH	7.4	7.4	7.4	7.4	7.4	7.4	7.4
Purity (%), time zero	95.1	95.8	95.7	95.6	95.9	95.9	95.7
Purity (%), 1 week 37°C	92.4	94.4	94.7	94.9	94.3	94.4	94.1
Purity (%), 1 week 37°C plus additional 6 days at 45°C	82.8	89.2	90.7	91.4	90.6	89.1	89.2
Purity loss (% points), 1 week 37°C	2.7	1.4	1.0	0.7	1.6	1.5	1.6
Purity loss (% points), 1 week 37°C plus additional 6 days at 45°C	12.3	6.6	5.0	4.2	5.3	6.8	6.5

Addition of 174 mM glycerol and 20 mM of the sugar alcohol sorbitol (Formulation 13) led to an increased stability as compared to 174 mM glycerol only (Formulation 7), although the stabilising effect was not as good as for the formulations comprising 174 mM glycerol and 20 mM mannitol (Formulation 10).

- 5 On the contrary, addition of 20 mM of the sugars sucrose (Formulation 14) or trehalose (Formulation 15) had no effect on stability, as can be seen when comparing with Formulation 7.

Table 2b. Effect on stability of various mannitol concentrations

Ingredients	Formulation				
	7	9	10	11	12
Insulin derivative of Example 1 (mM)	0.6	0.6	0.6	0.6	0.6
Zinc acetate (mM)	0.3	0.3	0.3	0.3	0.3
Mannitol (mM)	-	10	20	40	80
Glycerol (mM)	174	174	174	174	174
Phenol (mM)	16	16	16	16	16
Metacresol (mM)	16	16	16	16	16
Sodium chloride (mM)	10	10	10	10	10
Sodium phosphate (mM)	7	7	7	7	7
HCl	q.s.	q.s.	q.s.	q.s.	q.s.
NaOH	q.s.	q.s.	q.s.	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml
pH	7.4	7.4	7.4	7.4	7.4
Purity (%), time zero	95.8	95.7	95.6	95.7	95.9
Purity (%), 1 week 37°C	94.4	94.8	94.9	94.9	95
Purity (%), 1 week 37°C plus additional 6 days at 45°C	89.2	90.5	91.4	91.1	91.6
Purity loss (% points), 1 week 37°C	1.4	0.9	0.7	0.8	0.9
Purity loss (% points), 1 week 37°C plus additional 6 days at 45°C	6.6	5.2	4.2	4.6	4.3

Variation of the mannitol content (see Table 2b) showed that mannitol has a stabilising effect in a broad concentration range.

Example 5: Effect on stability from methionine in combination with mannitol

In this example the stabilizing effect of the combination of methionine and mannitol acid were investigated. In addition, the stability was determined at various pH levels. Chemical stability can be measured in terms of purity, where the content of the intact insulin derivative is determined at time zero and upon storage at a specific temperature for a specific time. The tested compositions are shown in Table 3.

Preparation process

Each formulation was prepared from the following stock solutions; 5 mM insulin derivative of Example 1, 100 mM sodium phosphate, 500 mM phenol, 160 mM metacresol, 1 M sodium chloride, 2.17 M glycerol, 10 mM zinc acetate, 335 mM methionine, 400 mM mannitol, water for injection.

The formulations were pH adjusted as necessary using HCl and NaOH and water for injection was added to reach the final desired volumes before being sterilised by filtration through a 0.22 µm sterile filter. After filtration, the formulations were filled into 3 ml sized drug cartridges to contain 0.5 ml of formulation storage stability.

Methods and assay experimentals

The cartridges were stored for 1 week at 45°C and analysed for purity which were compared to the results from analyses of the time point zero samples.

Purity (as percentage) for the insulin derivative of Example 1 was determined using UPLC equipped with a Waters column BEH C18, 2.1x150 mm, 1.7 µm, 130Å, gradient eluted over an approximately 70-minute run time using 0.1% TFA in milliQ water (buffer A) and 0.09% TFA in 90% ACN (buffer B) and using 9% acetonitrile as wash buffer. The column temperature was 50°C and the injection volume was 1 µl while the flow rate was set to 0.3 ml/min. Chromatography was analysed by UV detection (215 nm) and purity was evaluated as the area of the main peak divided by the area of all peaks x 100%.

Results

The purity (as percentages) at time zero and after storage for 1 week at 45°C for Formulations 16 to 21 are shown in Table 3. Purity loss is also provided as the difference (in percentage points) between the purity after storage and time zero.

Table 3. Effect on stability of combination of methionine and mannitol

Ingredients	Formulation					
	16	17	18	19	20	21
Insulin derivative of Example 1 (mM)	1.2	1.2	1.2	1.2	1.2	1.2
Zinc acetate (mM)	0.48	0.48	0.48	0.48	0.48	0.48
Methionine (mM)	-	-	-	20	20	20
Mannitol (mM)	20	20	20	20	20	20
Glycerol (mM)	217	217	217	217	217	217
Phenol (mM)	16	16	16	16	16	16
Metacresol (mM)	16	16	16	16	16	16
Sodium chloride (mM)	20	20	20	20	20	20
HCl	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
NaOH	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml
pH	7.2	6.8	6.4	7.2	6.8	6.4
Purity (%), time zero	95.0	95.0	94.8	95.2	95.1	94.9
Purity (%), 1 week 45°C	84.5	82.3	73.2	91.8	90.6	87.7
Purity loss (% points), 1 week 45°C	10.5	12.8	21.6	3.4	4.5	7.1

In Example 4 it was found that addition of glycerol and mannitol led to increased stability of the insulin formulation, and in Example 3 it was found that methionine increases stability. In this example, the purity loss at pH 7.2 for Formulation 16 comprising 217 mM glycerol and 20 mM mannitol was 10.5 percentage points after 1 week at 45°C. Addition of 20 mM methionine led to a surprisingly large increase in stability, with a purity loss under the same conditions of 3.4 percentage points (Formulation 19). The data in Table 3 also shows the effect of pH.

Example 6: Effect on stability from methionine and mannitol

In this example the stabilizing effect of methionine and mannitol in varying amounts on formulations of the insulin derivative of Example 1 was investigated. Chemical stability can be measured in terms of purity, where the content of the intact insulin derivative is

determined at time zero and upon storage at a specific temperature for a specific time. The tested compositions are shown in Table 4.

Preparation process

5 Each formulation was prepared from the following stock solutions; 8 mM insulin derivative of Example 1, 500 mM phenol, 160 mM metacresol, 1 M NaCl, 2.17 M glycerol, 10 mM zinc acetate, 100 mM methionine, 500 mM mannitol, water for injection.

The formulations were pH adjusted as necessary using HCl and NaOH and water was added to reach the final desired volumes before being sterilised by filtration through a
10 0.22 µm sterile filter. After filtration, the formulations were filled into HPLC vials to contain 50 µl for time zero analyses and filled into 3 ml sized drug cartridges to contain 0.5 ml of formulation for storage stability analyses.

Methods and assay experimental

15 The cartridges were stored for 4 weeks at 37°C and analysed for purity which were compared to the results from analyses of the time point zero samples.

Purity (as percentage) for the insulin derivative of Example 1 was determined using UPLC equipped with a Waters column BEH C18, 2.1x150 mm, 1.7 µm, 130Å gradient eluted over an approximately 64-minute run time using 0.1% TFA in milliQ water (buffer A) and
20 0.09% TFA in 90% ACN (buffer B) and using 9% acetonitrile as wash buffer. The column temperature was 35°C and the injection volume was 1 µl while the flow rate was set to 0.3 ml/min. Chromatography was analysed by UV detection (215 nm) and purity was evaluated as the area of the main peak divided by the area of all peaks x 100%.

25 Results

The purity (as percentages) at time zero and after storage for 2 weeks and 4 weeks, respectively, at 37°C for Formulations 22 to 27 are shown in Table 4. Purity loss is also provided as the difference (in percentage points) between the purity after storage and time
zero.

30

Table 4. Effect on stability of mannitol and methionine; alone and in combination

Ingredients	Formulation					
	22	23	24	25	26	27
Insulin derivative of Example 1 (mM)	1.2	1.2	1.2	1.2	1.2	1.2
Zinc acetate (mM)	0.48	0.48	0.48	0.48	0.48	0.48
Methionine (mM)	21	-	15	24	9	27
Mannitol (mM)	-	29	22	36	80	73
Glycerol (mM)	195	195	195	195	195	195
Phenol (mM)	18	18	18	18	18	18
Metacresol (mM)	18	18	18	18	18	18
Sodium chloride (mM)	20	20	20	20	20	20
HCl	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
NaOH	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml
pH	7.4	7.4	7.4	7.4	7.4	7.4
Purity (%), time zero	95.5	95.7	95.6	95.6	95.7	95.6
Purity (%), 2 weeks at 37°C	92.5	89.4	93.6	94.0	94.2	94.4
Purity (%), 4 weeks at 37°C	89.6	84.7	92.9	92.5	92.6	92.8
Purity loss, 2 weeks at 37°C	3.0	6.3	2	1.6	1.5	1.2
Purity loss, 4 weeks at 37°C	5.9	11.0	2.7	3.1	3.1	2.8

The data in Table 4 shows that the purity loss for Formulation 22 having 21 mM methionine and no mannitol was 5.9 percentage points after 4 weeks at 37°C. The purity loss for

5 Formulation 23 having 29 mM mannitol and no methionine was 11.0 percentage points. Formulations 24 and 25 with similar amounts of methionine and mannitol in combination surprisingly shows that the stability increases greatly by the combination of methionine and mannitol.

10 Example 7: Effect on stability from presence of zinc

In this example the impact from presence of zinc is shown for the insulin derivatives of Example 1 and Example 2. Chemical stability can be measured in terms of purity, where the content of the intact insulin derivative is determined at time zero and upon storage at a specific temperature for a specific time. The tested compositions are shown in Table 5.

Typically, insulin derivatives are more stable in the presence of zinc acetate and sodium chloride which for insulin is known to support a hexameric oligomeric state.

Preparation process

5 Each formulation was prepared from the following stock solutions; 1 mM insulin derivative of Example 1 or Example 2, 500 mM phenol, 160 mM metacresol, 1 M NaCl, 2.17 M glycerol, 10 mM zinc acetate, 168 mM methionine, 500 mM mannitol, water for injection.

The formulations were pH adjusted as necessary using HCl and NaOH and water was added to reach the final desired volumes before being sterilised by filtration through a
10 0.22 µm sterile filter. After filtration, the formulations were filled into HPLC vials to contain 50 µl. One sample was reserved and flash frozen for time zero analyses while another was used for storage stability for 1 week at 37°C before analysis.

Methods and assay experimental

15 The vials were stored for 1 week at 37°C and analysed for purity which was compared to the results from analyses of the time point zero samples.

Purity (as percentage) for both the insulin derivatives of Example 1 and Example 2 was determined using UPLC equipped with a Waters column BEH C18, 2.1x150 mm, 1.7 µm, 300Å, gradient eluted over an approximately 100-minute run time using 0.1% TFA in
20 milliQ water (buffer A) and 0.08% TFA in 80% ACN (buffer B) and using 9% acetonitrile as wash buffer. The column temperature was 55°C and around 8 µg was loaded onto the column while the flow rate was set to 0.3 ml/min. Chromatography was analysed by UV detection (215 nm) and purity was evaluated as the area of the main peak divided by the area of all peaks x 100%.

25

Results

The purity (as percentages) at time zero and after storage for 1 week at 37°C for Formulations 58 to 61 are shown in Table 5. Purity loss is also provided as the difference (in percentage points) between the purity after storage and time zero.

30

Table 5. Effect on stability of zinc

Ingredients	Formulation			
	58	59	60	61
Insulin derivative of Example 1 (mM)	0.6	0.6	-	-
Insulin derivative of Example 2 (mM)	-	-	0.6	0.6
Zinc acetate (mM)	0.24	-	0.24	-
Methionine (mM)	20	20	20	20
Mannitol (mM)	20	20	20	20
Glycerol (mM)	195	195	195	195
Phenol (mM)	16	16	16	16
Metacresol (mM)	16	16	16	16
Sodium chloride (mM)	20	20	20	20
HCl	q.s.	q.s.	q.s.	q.s.
NaOH	q.s.	q.s.	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml	To 1 ml	To 1 ml
pH	7.4	7.4	7.4	7.4
Purity (%), time zero	92.1	91.1	85.3	81.3
Purity (%), 1 weeks at 37°C	91.8	90.5	82.2	80.3
Purity loss, 1 weeks at 37°C	0.3	1.6	3.1	1.0

The data in Table 5 shows that for the insulin derivative of Example 1, the lowest purity loss is observed in the presence of zinc acetate and sodium chloride while for the insulin derivative of Example 2, surprisingly, the lowest purity loss is observed in the absence of zinc acetate and sodium chloride.

Example 8: Effect on stability from presence of zinc while varying pH and API concentration

In this example the impact from presence of zinc and varying pH is shown for two concentrations of the insulin derivative of Example 2. In addition, the impact of using sorbitol as tonicity agent instead of glycerol was tested. Chemical stability can be measured in terms of purity, where the content of the intact insulin derivative is determined at time zero and upon storage at a specific temperature for a specific time. The tested compositions are shown in Table 6a and Table 6b. Typically, insulin derivatives are more stable in the

presence of zinc acetate and sodium chloride which for insulin is known to support a hexameric oligomeric state.

Preparation process

5 Each formulation was prepared from the following stock solutions; 5.7 mM insulin derivative of Example 2, 500 mM phenol, 160 mM metacresol, 1 M NaCl, 2.17 M glycerol, 10 mM zinc acetate, 168 mM methionine, 500 mM mannitol, 1 M sorbitol, water for injection.

The formulations were pH adjusted as necessary using HCl and NaOH and water was added to reach the final desired volumes before being sterilised by filtration through a
10 0.22 µm sterile filter. After filtration, the formulations were filled into HPLC glass vials. One sample sized 50µl was flash frozen for time zero analyses while another sized 100µl was used for storage stability for 4 weeks at 37°C before analysis.

Methods and assay experimentals

15 The vials were stored for 1 week at 37°C and analysed for purity which was compared to the results from analyses of the time point zero samples.

Purity (as percentage) for both the insulin derivatives of Example 1 and Example 2 was determined using UPLC equipped with a Waters column BEH C8, 2.1x150 mm, 1.7 µm, 130Å, gradient eluted over an approximately 50-minute run time using 0.1% TFA in milliQ
20 water (buffer A) and 0.09% TFA in 80% ACN (buffer B), isocratic. The column temperature was 47°C and 0.3µl (samples diluted to 0.6mM insulin derivative of Example 2) was loaded onto the column while the flow rate was set to 0.3 ml/min. Chromatography was analysed by UV detection (214 nm) and purity was evaluated as the area of the main peak divided by the area of all peaks x 100%.

25

Results

The purity (as percentages) at time zero and after storage for 4 weeks at 37°C for Formulations 62-69 are shown in Table 6a. Purity loss is also provided as the difference (in percentage points) between the purity after storage and time zero.

30

Table 6a. Effect on stability of zinc and pH

Ingredients	Formulation							
	62	63	64	65	66	67	68	69
Insulin derivative of Example 2 (mM)	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Zinc acetate (mM)	0.24	0.24	0.24	0.24	-	-	-	-
Methionine (mM)	30	30	30	30	30	30	30	30
Mannitol (mM)	40	40	40	40	40	40	40	40
Glycerol (mM)	185	185	185	185	185	185	185	185
Phenol (mM)	16	16	16	16	16	16	16	16
Metacresol (mM)	16	16	16	16	16	16	16	16
Sodium chloride (mM)	20	20	20	20	-	-	-	-
HCl	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
NaOH	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml
pH	6.6	7.0	7.4	7.8	6.6	7.0	7.4	7.8
Purity (%), time zero	95.2	94.4	93.8	94.3	95.2	94.5	94.8	94.3
Purity (%), 4 weeks at 37°C	82.0	85.0	86.2	84.8	89.8	89.4	88.9	85.5
Purity loss, 4 weeks at 37°C	13.2	9.4	7.6	9.5	5.4	5.1	5.9	8.8

The data in Table 6a shows that for the insulin derivative of Example 2, surprisingly, the lowest purity loss is observed in the absence of zinc acetate and sodium chloride and near pH 6.6.

Table 6b. Effect on stability of sorbitol vs glycerol

Ingredients	Formulation	
	70	71
Insulin derivative of Example 2 (mM)	1.2	1.2
Zinc acetate (mM)	-	-
Methionine (mM)	30	30
Mannitol (mM)	40	40
Sorbitol (mM)		185
Glycerol (mM)	185	
Phenol (mM)	16	16
Metacresol (mM)	16	16
HCl	q.s.	q.s.
NaOH	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml
pH	7.4	7.4
Purity (%), time zero	95.3	95.4
Purity (%), 4 weeks at 37°C	87.4	88.2
Purity loss, 4 weeks at 37°C	7.9	7.2

From the data in Table 6b, it can be seen that replacing glycerol with sorbitol leads to a formulation with similar stability.

5

Example 9: Effect on stability from various excipients

In this example, formulations with varying amounts of the insulin derivative of Example 2, varying amounts of glycerol and mannitol, varying amounts of the antioxidant methionine and varying pH were prepared, and the stability was tested. Chemical stability can be measured in terms of purity, where the content of the intact insulin derivative is determined at time zero and upon storage at a specific temperature for a specific time. The tested compositions are shown in Table 7a, Table 7b, Table 7c and Table 7d.

10

Preparation process

Each formulation was prepared from the following stock solutions; 5.7 mM insulin derivative of Example 1, 500 mM phenol, 160 mM metacresol, 1 M sodium chloride, 2.17 M glycerol, 10 mM zinc acetate, 167.5 mM methionine, 500 mM mannitol, 1000 mM sorbitol, water for injection.

15

The formulations were pH adjusted as necessary using HCl and NaOH and water for injection was added to reach the final desired volumes before being sterilised by filtration through a 0.22 µm sterile filter. After filtration, the formulations were filled into flatbottomed glass UPLC vials to contain 0.3 ml of formulation for storage stability.

5

Methods and assay experimentals

Vials were stored for 4 weeks at 37°C and analysed for purity which were compared to the results from analyses of the time point zero samples.

Purity (as percentage) for the insulin derivative was determined using UPLC equipped with a Waters column BEH C8, 2.1x150 mm, 1.7 µm, 130Å, gradient eluted over an approximately 50-minute run time using 0.1% TFA in milliQ water (buffer A) and 0.09% TFA in 90% ACN (buffer B) and using 9% acetonitrile as wash buffer. The column temperature was 47°C and the injection volume was 0.3 µl for samples containing 0.6 mM insulin derivative while the flow rate was set to 0.3 ml/min. Samples containing 1.2 mM insulin derivative were diluted to 0.6 mM using water immediately prior to loading. Chromatography was analysed by UV detection (214 nm) and purity was evaluated as the area of the main peak divided by the area of all peaks x 100%.

Results

The purity (as percentages) at time zero and after storage for 4 weeks at 37°C for Formulations 28 to 38 are shown in Table 7a, Table 7b, Table 7c and Table 7d. Purity loss is also provided as the difference (in percentage points) between the purity after storage and time zero.

By comparing formulations 28-30 (Table 7a), it can be seen that the stability is decreased if the pH is lowered to 5.8, as compared to when the pH is 6.2 and 6.6, respectively.

It can be seen from Formulations 32, 34, 35 and 36 (Table 7b) that a stable formulation can be achieved with a various concentrations of methionine, mannitol and glycerol. It appears that the most stable formulation is achieved by a methionine concentration of at least 30 mM and a mannitol concentration of at least 40 mM.

It can be seen from Formulations 32 and 33 (Table 7c) that a stable formulation can be achieved using the preservatives phenol and metacresol alone and in combination.

35

Table 7a. Effect on stability of pH

Ingredients	Formulation		
	28	29	30
Insulin derivative of Example 2 (mM)	0.6	0.6	0.6
Methionine (mM)	30	30	30
Mannitol (mM)	40	40	40
Glycerol (mM)	217	217	217
Phenol (mM)	16	16	16
Metacresol (mM)	16	16	16
HCl	q.s.	q.s.	q.s.
NaOH	q.s.	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml	To 1 ml
pH	5.8	6.2	6.6
Purity (%), time zero	88.6	89.3	89.7
Purity (%), 4 weeks at 37°C	83.2	86.0	86.5
Purity loss, 4 weeks at 37°C	5.4	3.3	3.2

Table 7b. Effect on stability of methionine and mannitol concentrations

Ingredients	Formulation			
	32	34	35	36
Insulin derivative of Example 2 (mM)	2.4	2.4	2.4	2.4
Methionine (mM)	30	15	30	30
Mannitol (mM)	40	40	80	20
Glycerol (mM)	217	217	174	195
Phenol (mM)	16	16	16	16
Metacresol (mM)	16	16	16	16
HCl	q.s.	q.s.	q.s.	q.s.
NaOH	q.s.	q.s.	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml	To 1 ml	To 1 ml
pH	6.6	6.6	6.6	6.6
Purity (%), time zero	90.0	89.9	89.9	90.3
Purity (%), 4 weeks at 37°C	86.4	85.9	86.3	85.6
Purity loss, 4 weeks at 37°C	3.6	4.0	3.6	4.7

Table 7c. Effect on stability of preservatives

Ingredients	Formulation	
	32	33
Insulin derivative of Example 2 (mM)	2.4	2.4
Methionine (mM)	30	30
Mannitol (mM)	40	40
Glycerol (mM)	217	217
Phenol (mM)	16	-
Metacresol (mM)	16	32
HCl	q.s.	q.s.
NaOH	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml
pH	6.6	6.6
Purity (%), time zero	90.0	89.9
Purity (%), 4 weeks at 37°C	86.4	85.8
Purity loss, 4 weeks at 37°C	3.6	4.1

Table 7d. Effect on stability of various concentrations of insulin derivative

Ingredients	Formulation				
	30	31	32	37	38
Insulin derivative of Example 2 (mM)	0.6	1.2	2.4	3.6	4.8
Methionine (mM)	30	30	30	30	30
Mannitol (mM)	40	40	40	40	40
Glycerol (mM)	217	217	217	217	217
Phenol (mM)	16	16	16	16	16
Metacresol (mM)	16	16	16	16	16
HCl	q.s.	q.s.	q.s.	q.s.	q.s.
NaOH	q.s.	q.s.	q.s.	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml
pH	6.6	6.6	6.6	6.6	6.6
Purity (%), time zero	89.7	89.2	90.0	90.0	89.8
Purity (%), 4 weeks at 37°C	86.5	86.6	86.4	85.8	86.4
Purity loss, 4 weeks at 37°C	3.2	2.6	3.6	4.2	3.4

In formulations 30, 31, 32, 37 and 38 (Table 7d), the concentration of the insulin derivative is varied from 0.6 mM to 4.8 Mm, and it can be seen that a stable formulation can be achieved over the entire concentration range.

5 Example 10: Effect on stability from various excipients

In this example, formulations containing the insulin derivative of example 2 were prepared to contain varying amounts of preservative agents, methionine, mannitol and glycerol, and the stability was tested. Chemical stability can be measured in terms of formation of High Molecular Weight Products (HMWP), where the content of HMWP is determined at time zero and upon storage at a specific temperature for a specific time. The tested compositions are shown in Table 8a, Table 8b, and Table 8c.

Preparation process

Each formulation was prepared from stock solutions of insulin derivative of Example 2 and excipients as shown in Table 8a, Table 8b and Table 8c. The formulations were pH adjusted as necessary using either HCl and NaOH or acetic acid and water for injection was added to reach the final desired volumes before being sterilised by filtration through a 0.22 µm sterile filter. After filtration, the formulations were filled into HPLC vials for storage stability.

Methods and assay experimentals

Vials were stored for 6 weeks at 37°C and analysed for presence of high molecular weight proteins (HMWP) which were compared to the results from analyses of the time point zero samples.

HMWP (as percentage) for the insulin derivative was determined using SE-UPLC equipped with a Waters Acquity BEH SEC Insulin column, 4.6x150 mm, 1.7 µm, 125Å, isocratic elution over an approximately 10-minute run time using 300 mM sodium chloride, 10 mM sodium phosphate, 5 mM phosphoric acid, 15% isopropanol and 35% ACN. The column temperature was 50°C and the injection volume was 0.6 µl for samples diluted to contain 0.24 mM insulin derivative from Example 2 (10x) while the flow rate was set to 0.3 ml/min. Chromatography was analysed by UV detection (214 nm) and percentage HMWP was evaluated as the area of the HMWP peak in relation to the total peak area (100%).

Results

The tested compositions for Formulations 39 to 57 are shown in Table 8a, Table 8b, and Table 8c along with the results on HMWP (as percentages) at time zero and after storage for 6 weeks at 37°C.

5

Table 8a. Effect on stability of mannitol and methionine; alone and in combination

Ingredients	Formulation				
	39	40	41	42	57
Insulin derivative of Example 2 (mM)	2.4	2.4	2.4	2.4	2.4
Methionine (mM)	-	-	30	30	30
Mannitol (mM)	-	40	-	40	40
Glycerol (mM)	185	185	185	185	165
Phenol (mM)	25	25	25	25	25
Metacresol (mM)	25	25	25	25	25
HCl	q.s.	q.s.	q.s.	q.s.	q.s.
NaOH	q.s.	q.s.	q.s.	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml
pH	6.6	6.6	6.6	6.6	6.6
HMWP (%), time zero	0.98	0.99	0.99	0.99	1.03
HMWP (%), 6 weeks at 37°C	3.06	2.58	2.62	1.93	1.92
ΔHMWP formation (%), 6 weeks at 37°C	2.08	1.59	1.63	0.94	0.89

It can be seen from Table 8a that the formation of HMWP during 6 weeks storage was found to be lower for formulations containing either methionine or mannitol as compared to Formulation 39 without. Formulations with both methionine and mannitol had even lower HMWP formation.

10

15

In formulations 42, 49, 50, 51, and 52 (Table 8b) the concentration of methionine is varied from 15 mM to 45 mM, and the concentration of mannitol is varied from 20 mM to 80 Mm, and it can be seen that a stable formulation can be achieved over the entire concentration ranges.

5

Table 8b. Effect on stability of various concentrations of methionine and mannitol

Ingredients	Formulation				
	42	49	50	51	52
Insulin derivative of Example 2 (mM)	2.4	2.4	2.4	2.4	2.4
Methionine (mM)	30	45	15	30	30
Mannitol (mM)	40	40	40	80	20
Glycerol (mM)	185	185	185	185	185
Phenol (mM)	25	25	25	25	25
Metacresol (mM)	25	25	25	25	25
HCl	q.s.	q.s.	q.s.	q.s.	q.s.
NaOH	q.s.	q.s.	q.s.	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml
pH	6.6	6.6	6.6	6.6	6.6
HMWP (%), time zero	0.99	1.01	1.01	1.01	1.00
HMWP (%), 6 weeks at 37°C	1.93	1.82	2.15	1.89	2.03
ΔHMWP formation (%), 6 weeks at 37°C	0.94	0.81	1.14	0.88	1.02

10 It can be seen from Formulations 42, 43, 44, 45, 46, 47 and 48 (Table 8c) that a stable formulation can be achieved using the preservatives phenol and metacresol alone and in combination in various concentrations.

15

Table 8c. Effect on stability of preservatives

Ingredients	Formulation						
	42	43	44	45	46	47	48
Insulin derivative of Example 2 (mM)	2.4	2.4	2.4	2.4	2.4	2.4	2.4
Methionine (mM)	30	30	30	30	30	30	30
Mannitol (mM)	40	40	40	40	40	40	40
Glycerol (mM)	185	185	185	185	185	185	185
Phenol (mM)	25	21	16	-	16	60	60
Metacresol (mM)	25	21	16	32	32	16	-
HCl	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
NaOH	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Water for injection	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml	To 1 ml
pH	6.6	6.6	6.6	6.6	6.6	6.6	6.6
HMWP (%), time zero	0.99	0.99	1.00	0.98	1.00	1.01	1.02
HMWP (%), 6 weeks at 37°C	1.93	1.92	1.82	1.87	1.93	2.00	1.89
ΔHMWP formation (%), 6 weeks at 37°C	0.94	0.93	0.82	0.89	0.93	0.99	0.87

Example 11: Stability data for a formulation from insulin derivative of Example 2

5 This example describes the long-term stability data for a formulation of insulin derivative of Example 2. Chemical stability can be measured in terms of purity, where the content of the intact insulin derivative is determined at time zero and upon storage at a specific temperature for a specific time. The tested composition is shown in Table 9.

10 Preparation process

 The formulation was prepared from crude pharmacopeia grade excipients, phenol, metacresol, mannitol, methionine, glycerol and water for injection. An excipient formulation was prepared at 80% of the final volume wherein insulin derivative of Example 2 was dissolved. The formulation was pH adjusted as necessary using HCl and NaOH and water
15 for injection was added to reach the final desired volumes before being sterilised by filtration

through a 0.22 µm sterile filter. After filtration, the formulations were filled into glass penfills to contain 50 µl for time zero analyses and filled into 3 ml sized drug cartridges to contain 1 ml of formulation for storage stability analyses.

5 Methods and assay experimentals

A penfill was analysed at time zero while remaining penfills were stored for up to 6 months at 5°C and 25°C as well as up to 3 months at 30°C and 37°C whereafter the purity was analysed. The results were compared to the results from analyses of the time point zero samples.

- 10 Purity (as percentage) for the insulin derivative of Example 2 was determined using UPLC equipped with a Waters column BEH C8, 2.1x150 mm, 1.7 µm, 130Å, gradient eluted over an approximately 40 minute run time using 0.1% TFA in milliQ water (buffer A) and 0.09% TFA in 90% ACN (buffer B). The column temperature was 41°C and around 8µg was loaded onto the column while the flow rate was set to 0.3 ml/min. Chromatography was
- 15 analysed by UV detection (214 nm) and purity was evaluated as the area of the main peak divided by the area of all peaks x 100%.

Results

- The purity (as percentages) at time zero and after storage for up to 6 months at 5°C
- 20 and 25°C as well as up to 3 months at 30°C and 37°C for Formulation 72 is shown in Table 9. Purity loss is also provided as the difference (in percentage points) between the purity after storage and time zero.

- 25 The data in Table 9 shows the stability of a formulation of the invention after 3 months and after 6 months at 5°C, 25°C, 30°C, and 37°C, respectively.

Table 9. Stability of a formulation of the invention

Ingredients	Formulation
	72
Insulin derivative of Example 2 (mM)	1.2
Methionine (mM)	30
Mannitol (mM)	40
Glycerol (mM)	152
Phenol (mM)	25
Metacresol (mM)	25
HCl	q.s.
NaOH	q.s.
Water for injection	To 1 ml
pH	6.6
Purity (%), Time zero	90.8
Purity (%), 6 months at 5°C	90.8
Purity (%), 6 months at 25°C	84.4
Purity (%), 3 months at 30°C	83.1
Purity (%), 3 months at 37°C	72.6
Purity loss, 6 months at 5°C	0.0
Purity loss, 6 months at 25°C	6.4
Purity loss, 3 months at 30°C	7.7
Purity loss, 3 months at 37°C	18.2

- 5 While certain features of the invention have been illustrated and described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the invention.

CLAIMS

1. A liquid pharmaceutical composition comprising
an insulin derivative comprising at least one aryl boron moiety, such as an aryl
boronic acid or aryl boroxol; and
methionine.
2. The liquid pharmaceutical composition according to claim 1, further comprising
glycerol or a sugar alcohol, such as mannitol or sorbitol, either alone or in
combination.
3. The liquid pharmaceutical composition according to claim 2, comprising mannitol.
4. The liquid pharmaceutical composition according to claim 2, comprising mannitol and
glycerol.
5. The liquid pharmaceutical composition according to any one of claims 1 to 4, wherein
the pH is within the range of 5.0 to 8.0.
6. The liquid pharmaceutical composition according to any one of claims 1 to 5, further
comprising a phenolic preservative or a mixture of phenolic preservatives.
7. The liquid pharmaceutical composition according to any one of claims 1 to 6, wherein
the insulin derivative is the insulin derivative of Example 1, or a pharmaceutically
acceptable salt thereof, or the insulin derivative of Example 2, or a pharmaceutically
acceptable salt thereof.
8. The liquid pharmaceutical composition according to any one of claims 1 to 7, wherein
the composition comprises:
the insulin derivative of Example 2, or a pharmaceutically acceptable salt thereof,
glycerol,
mannitol, and
methionine.

9. The liquid pharmaceutical composition according to claim 8, wherein the composition is essentially free of zinc.
10. The liquid pharmaceutical composition according to any one of claims 1 to 9,
5 wherein the composition comprises:
0.6 to 4.8 mM of the insulin derivative of Example 2, or a pharmaceutically acceptable salt thereof,
30 mM methionine,
152 mM glycerol,
10 40 mM mannitol,
25 mM phenol,
25 mM metacresol,
and wherein the pH is 6.6.
11. The liquid pharmaceutical composition according to claim 10, wherein the
15 composition comprises:
1.2 mM of the insulin derivative of Example 2, or a pharmaceutically acceptable salt thereof,
30 mM methionine,
20 152 mM glycerol,
40 mM mannitol,
25 mM phenol,
25 mM metacresol,
and wherein the pH is 6.6.
12. A liquid pharmaceutical composition comprising:
1.2 mM of the insulin derivative of Example 2, or a pharmaceutically acceptable salt thereof,
30 mM methionine,
30 152 mM glycerol,
40 mM mannitol,
25 mM phenol,
25 mM metacresol,
and wherein the pH is 6.6.

35

13. A liquid pharmaceutical composition according to any one of claims 1 to 12, for use as a medicament.
- 5 14. A liquid pharmaceutical composition according to any one of claims 1 to 12, for use in the prevention or treatment of diabetes, including diabetes of Type 1 and diabetes of Type 2.
- 10 15. A method for the treatment of diabetes, such as diabetes of type 1 and diabetes of type 2, in a subject comprising administering to said subject a liquid pharmaceutical composition according to any one of claims 1 to 12.