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[^{125}I]- and [^{14}C]- Labelling of the Long-Acting Insulin Derivative NN304.

Jesper B. Kristensen, Lars K. Müller, Ulla D. Larsen,
Louis B. Hansen and Christian Foged
Novo Nordisk A/S DK-2760 Måløv Denmark

Summary: Lys$^{\varepsilon B29}$ Tetradecanoyl des(B30) human insulin, Novo Nordisk code no NN304, is a long-acting human insulin derivative that differs from human insulin by being more than 98% albumin bound in plasma. The syntheses of [^{125}I]Tyr^{A14} N$^{\varepsilon}$-Lys$^{\varepsilon B29}$ Tetradecanoyl des(B30) human insulin ([^{125}I]NN304) and Lys$^{\varepsilon B29}$ [1-^{14}C]-Tetradecanoyl des(B30) human insulin ([^{14}C]NN304) used for absorption, distribution, metabolism and excretion (ADME) studies are described. NN304 was radio-iodinated using the lactoperoxidase/hydrogen peroxide method, and the product was isolated using RP-HPLC. Radio-peptide mapping confirmed the [^{125}I] labelling position at Tyrosine 14 in the A-chain. The radiochemical yield was 30% with a radiochemical purity of >98%. [^{14}C]-NN304 was synthesised in two steps starting from 1-[^{14}C] myristic acid and subsequently purified by HPLC. The overall radiochemical yield was 15% with a radiochemical purity >98%.

Key words: Iodine-125, Carbon-14, insulin derivative (NN304), radio-peptide mapping.

Introduction

Lys$^{\varepsilon B29}$ Tetradecanoyl des(B30) human insulin (NN304) (Figure 1) is a long-acting insulin derivative that differs from human insulin by being more than 97% albumin bound in plasma [1, 2]. The high affinity (1.0 x 10^{-5} M^{-1}) for serum albumin is caused by the fatty acid attaching itself the ε-amino group of Lys B29[3]. The albumin binding slows the transport of NN304 from the injection site (subcutaneous) leading to an increase in plasma half life[4]. To be able to perform ADME studies on NN304, a radiolabelled form of the compound was needed. Peptides are normally radiolabelled with [^{125}I] for ADME studies, but since NN304 also contains a tetradecanoyl group of interest, it was decided to

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label the molecule with both [^{125}I] and [^{14}C] in the peptide and fatty acid part of the molecule respectively. The two different radiolabelled positions would allow a more detailed study of the compound in the organism.

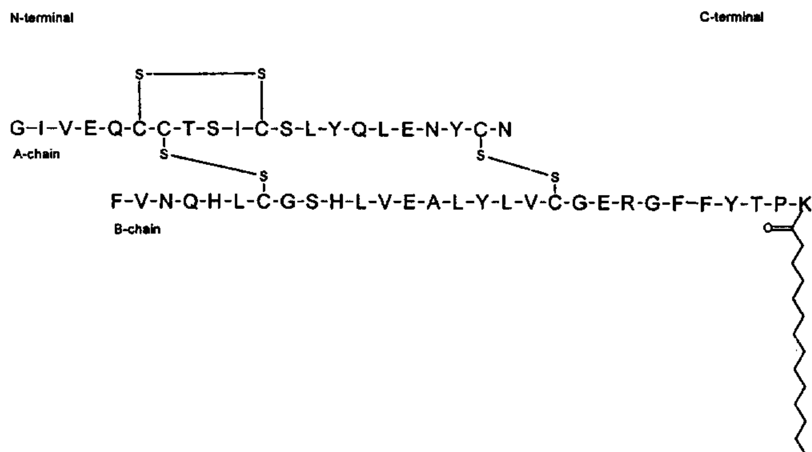

Figure 1: Structure of NN304

Results and Discussion

[^{125}I]Tyr^{A14} Lys$^{\varepsilon B29}$ Tetradecanoyl des(B30) human insulin.

NN304 has tyrosine in position A14, A19, B16 and B26 as possible iodination sites. Labelling of NN304 in tyrosine A14 was chosen since it has previously been shown that [^{125}I]Tyr A14 insulin[5] and [^{125}I]Tyr A14 insulin analogues[6] retain their full biological activity compared to unlabelled reference material.

For iodination, the lactoperoxidase method was selected. This method gives a low degree of peptide degradation and a high incorporation of iodine in the Tyr A14 position of insulin and insulin analogues[6]. The amount of oxidising reagent (hydrogen peroxide) used was equimolar to the amount of [^{125}I]. This means that the addition of a reducing reagent to stop the reaction (which could be harmful to the sulphur bridges in insulin) was avoided. The molar amount of NN304 relative to [^{125}I] was kept above 100:1 in order to avoid the formation of double-labelled NN304 molecules.

The RP-HPLC elution profile for human insulin labelled by the lactoperoxidase method is comparable to the one shown for the [^{125}I]NN304 iodination

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mixture (Figure 2). In the case of [^{125}I] labelled human insulin the radioactive material eluting first and last was identified as [^{125}I]Tyr A19 human insulin and [^{125}I]Tyr A14 human insulin respectively[6]. We therefore presumed that the peak eluting last was [^{125}I]Tyr A14 NN304.

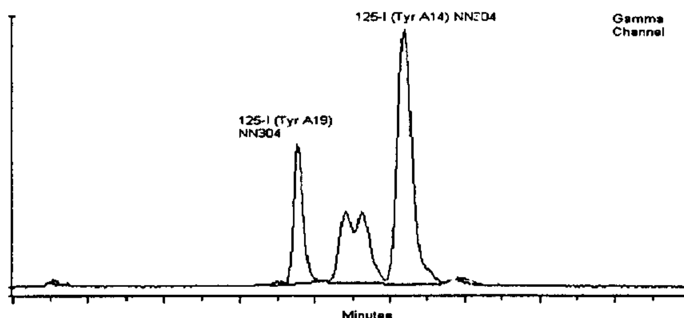

Figure 2: Preparative HPLC chromatogram of NN304 iodination mixture.

To confirm this radio-peptide mapping was performed. Peptide mapping by HPLC is a useful technique for defining the primary sequence of proteins[7] . It involves enzymatic digestion of the target protein, whereby specific fragments are obtained whose numbers and properties are dependent upon the protein under investigation. These fragments can then be "mapped" by a suitable chromatographic method. In the case of NN304, digestion of the parent molecule is achieved with the use of *Staph. Aureus* V8 protease enzyme, which cleaves the C-terminal of glutamic acid[7]. Thus, after digestion NN304 is cleaved into four fragments (Figure 3) and these fragments can be identified by RP-HPLC. The chromatogram of protease treated NN304, includes the four fragments in addition to a limited amount of undigested NN304 and enzyme. The peaks have previously been identified (Novo Nordisk unpublished results) as shown on the chromatogram (UV) (Figure 4). The chromatogram in Figure 4 shows enzyme digested NN304 spiked with [^{125}I]Tyr A14 NN304.

The chromatogram shows two radiochemical peaks with retention times of 20.0 min and 30.4 min. The latter small peak represents undigested tracer, which was confirmed by a control run. The main radioactivity peak at 20,0 min corresponds to fragment I (The radioactivity peak has a 2 minute longer retention-time due to the presence of the iodine in the fragment).

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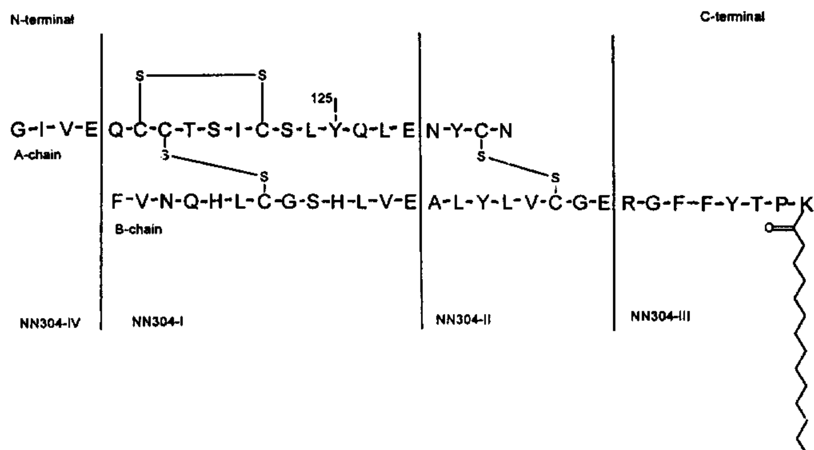

Figure 3: Structure of NN304 fragments. (NN304 digested by *Staph. Aureus* V8 protease enzyme).

A similar time delay was seen with NN304 labelled in Tyrosine A19. Tyrosine A19 is located in fragment NN304-II which has a retention time of approx. 15 min. The chromatogram of the digested sample containing [^{125}I]Tyr A19 NN304 contains two radiochemical peaks with retention times of 17 min. and 30 min (Figure 5).

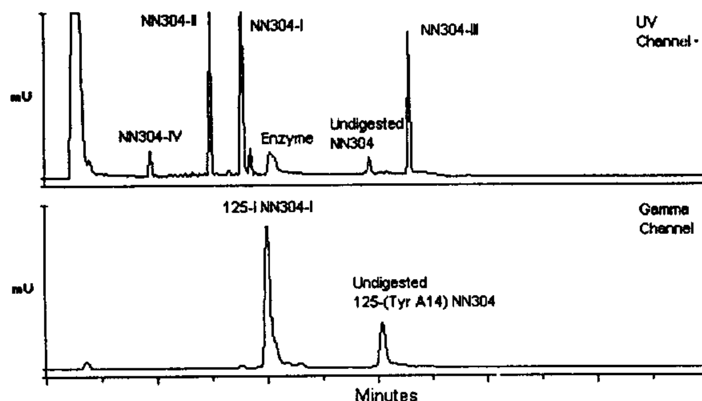

Figure 4: HPLC of enzyme digested NN304 spiked with [^{125}I](Tyr A14) NN304 (UV and gamma detection).

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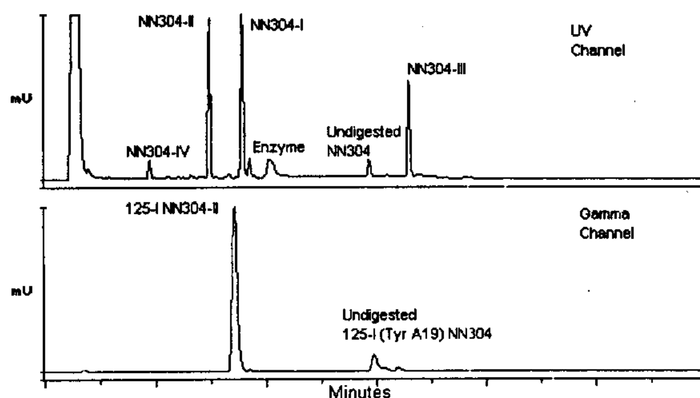

Figure 5: HPLC of enzyme digested NN304 spiked with [^{125}I]Tyr A19 NN304 (UV and gamma detection).

The radio-peptide mapping experiment confirmed the expected labelling position of the two purified isomers.

Lys$^{\varepsilon B29}$ 1-[^{14}C]-Tetradecanoyl des(B30) human insulin

Lys$^{\varepsilon B29}$ 1-[^{14}C]-Tetradecanoyl des(B30) human insulin was synthesised using the commercially available radioactive substance [1-^{14}C]myristic acid as starting material.

The selectivity of the coupling of [1-^{14}C]myristic acid with des(B30) human insulin, can be controlled by using the reagent [1-^{14}C]tetradecanoat N-hydroxysuccinimide ester under alkaline conditions[8, 9]. This reagent was prepared by reacting (1) with (2) in the presence of a dehydrating agent, N,N′-dicyclohexylcarbodiimide (DCC).

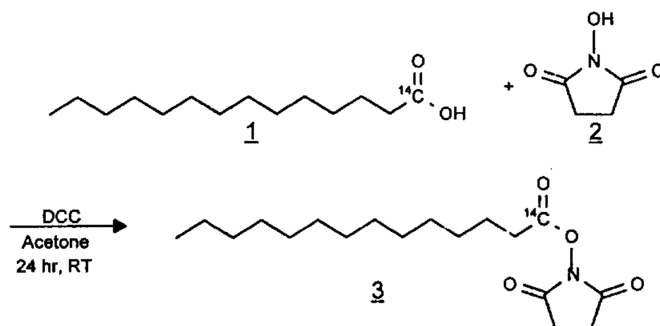

Figure 6: Reaction of [1-^{14}C]myristic acid (1) and N-hydroxysuccinimide (2).

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The coupling between (3) and des(B30) human insulin was carried out in a mixture of 1-methyl-2-pyrrolidone and water using N-ethyldiisopropylamine as the base.

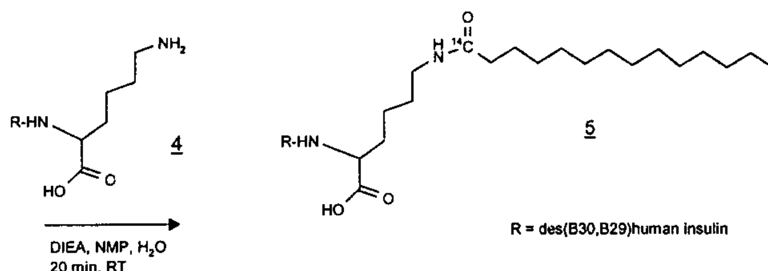

Figure 7: reaction of [1-^{14}C]tetradecanoate N-hydroxysuccinimide ester (3) and des(B30) human insulin (4).

To reduce impurities from the reaction mixture, a two-step purification procedure was performed. The first step was ion-exchange chromatography, which removed more than 90% of the impurities. However, to obtain a radiochemical purity >98% a subsequent reverse phase purification on a C-4 column was needed. The product from the preparative HPLC was worked up using a C-18 SepPak. The use of SepPak provided a gentle way to concentrate the product in a salt-free solution.

The [^{14}C]NN 304 (100 μCi/ml) was formulated in 70% EtOH containing 0.1M TRIS and 0.05M phosphoric acid, pH 7.5. The specific radioactivity for [^{14}C]NN 304 was determined to be 56 mCi/mmol using HPLC and reference material. The overall radiochemical yield was 15% corresponding to 1.5 mCi.

Experimental

Chemicals

Chemicals were purchased from Merck, Fluka or Aldrich and used without further purification. Lactoperoxidase was from Sigma. All reagents and solvents used were of analytical grade, all water used was produced through a Millipore filtration system. [1-^{14}C]Myristic acid (code CFQ.9237) was supplied by Amersham International plc, UK, dissolved in ethanol (1.67 mCi / ml, specific radioactivity 56 mCi/mmol). Na^{125}I (Amersham, UK, Code No. IMS 30).

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Des (B30) human insulin and NN304 was supplied by Novo Nordisk A/S.

HPLC systems used for $[^{125}I]$

HPLC purifications and analysis were performed using a Merck HPLC pump L-6200 with a rheo-dyne injector (500 μl loop, 100 μl for analytical systems) and a 250 mm x 4.0 mm, C18, 120 Å, 5 μm (Novo Nordisk A/S) column. A Merck Hitachi Detector UV L-4250 and Merck Hitachi Column Thermostat and Merck Hitachi Interface D-6000 were used. For radioactivity detection a gamma-analyser model 45-24, Moelsgaard Medical A/S, Denmark and a Harshaw/Filtrol Scintillation NaI/Tl crystal was used (preparative) and a Berthold HPLC radioactivity monitor LB 507B (J 1000 gamma cell, loop volume 150 μl) (analytical). HPLC methods are shown in Table 1.

Table 1: HPLC methods

Method	Column	Mobile phase	Gradient
$[^{125}I]$ Preparative/analysis C18	NN RP C18 OdOMeSi 5μm (YMC 120Å) 4 x 250 mm	A: 0.1 M Tris, 0.5M H_3PO_4, 49% EtOH Flow: 0.5 ml/min.	Isocratic
$[^{125}I]$ Pep-map C8	Zorbax C8 RP (4.6 x 25 mm) part no. 880952706	A: 0.53% $(NH_4)_2SO_4$, 048% H_2SO_4, 7.9% ACN B: 0.58% $(NH_4)_2SO_4$, 053% H_2SO_4, 52.1% ACN Flow: 1 ml/min	0-100% B 0-30 min.
$[^{14}C]$ analysis C18	NN RP C18 OdOMeSi 5 μm(YMC 120AA) 4 x 250 mm (60°C) (UV 214)	A: 0.1% TFA 10% ACN B: 0.1% TFA 90% ACN Flow: 1 ml/min.	45%-75% B 0-60 min. 75%-100% B 60-65 min.
$[^{14}C]$ Preparative Ion-exc.	Pharmacia type source 30Q NN 10 x 250 mm (20 °C) (UV 280)	A: 0.24% Tris, 42% EtOH B: 0.24% Tris, 42% EtOH, 1% NH_3CH_3COOH C: 12.1% Tris, 42% EtOH Flow: 2 ml/min.	0%-100% B 0-200 min. 100%C 200-240 min.
$[^{14}C]$ Preparative C4	NN RP C4 Bu-DMeSi (15μm) (YMC 300AA) 10 x 250 mm. (20 °C) (UV 276)	A: 0.63% citric acid, 0.74% KCl, 32 % EtOH. B: 0.63% citric acid, 0.74% KCl, 38 % EtOH C: 1M Acetic acid, 70% EtOH Flow: 2 ml/min.	0%-100 % B 0-250 min. 100% C 250-280 min

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HPLC systems used for [^{14}C]

HPLC analyses and semi-preparative purifications were performed using a Merck HPLC pump L-6200A with a rheo-dyne injector (100 µl loop for analysis, 5000 µl loop for semi-preparative purifications) and a Merck UV-detector L-4000A (operating at 214nm or 280nm). For radioactive detection during HPLC-analysis a Canberra Packard Flow Detector A-500 was used.

Radioactivity in the column effluent during HPLC-analysis was monitored with a Radiomatic/Canberra Flo-One beta detector, using a 500 µl liquid flow cell. The ratio of column effluent to liquid scintillator (Ultima-flow M™, Packard) was 1:2. HPLC methods are shown in Table 1.

Radioactivity Counting

For determination of total radioactivity in [^{125}I] samples a Gamma-analyser, model 45- 24, Moelsgaard Medical A/S, Denmark, Scaler-timer, model 47-22, Moelsgaard Medical A/S, Denmark, Harshaw/Filtrol Scintillation NaI/Tl crystal was used. For determination of total radioactivity in [^{14}C] samples a Packard 1000 TR tricarb liquid scintillation analyser was used, using 20 ml counting vials and Ultima-flow M™ liquid scintillator supplied by Packard DK.

Thin Layer Chromatography

TLC was performed on glass plates coated with silica gel 60 (5 x 20 cm, Merck Art 1.05714). The mobile phase consisted of a mixture of hexane : ethylacetate : acetic acid (80:10:1 [v/v/v]). Radio-TLC analyses were performed using a Bioscan Imaging Scanner System 200-IBM with an Autochanger 1000. The collimator grid contained 10 strings/mm and the P10 gas (10% methane in argon) flow was 1.5 l/min.

Chemistry [^{125}I]NN304.

A 0.7 mM (4 mg/ml) neutral solution of NN304 was prepared by dissolving 3.8 mg (0.64 µmol) of insulin NN304 in 940 µl of H_2O.

Radioiodination was performed as follows: 100 µl of a 0.7 mM (70 nmol) insulin NN304 solution was incubated with 4 mCi, (1.8 nmol), 20 µl 0.33 mM H_2O_2 and 20 µl of a 0.1 mg/ml solution of lactoperoxidase. The solution was

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incubated for 5 minutes. The incorporation of [125I] was determined by precipitation of an aliquot (0.5 µl) with TCA.

Prior to application on RP-HPLC the iodination mixture was adjusted to 500 µl with HPLC-buffer. Elution was performed isocratically at 0.5 ml/min at 40°C with 0.1 M Tris, 0.05 M H_3PO_4 (pH 7.5) containing 49% ethanol. The radioactivity was detected online and the peak corresponding to the [125I]Tyr A14 NN304 was collected using a fraction collector.

The collected HPLC fraction was adjusted with water (HPLC grade) and ethanol to a formulation containing 100 µCi [125I]Tyr A14 NN304/ml in 70 % ethanol.

Peptide Mapping

Before sample preparation most of the ethanol (>75% of the original volume) was evaporated from the tracer sample and the tracer controls using a vacuum centrifuge. The samples were diluted to approx. 1 µCi/µl by adding 0,1 M 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid: Merck Art.110110), pH 7.5 adjusted with potassium hydroxide (Hepes buffer). The samples that contained tracer solutions all included the same concentration of reference substance (cold insulin NN304). This insured that all the enzymatic digestions were performed under similar conditions and it provided a built in control for each run whereby it was possible to assess whether or not the enzymatic reaction had proceeded, simply by assessment of the HPLC chromatogram (UV).

Samples were prepared and processed by pipetting the following into 75 mm x 11.5 mm sample tubes: See Table 2.

Table 2: Sample composition for radio peptide mapping

Sample type	Sample composition
Substrate blind (no enzyme)	100 ul Reference substance (NN304 2 mg/ml dissolved in Hepes buffer), 400 µl Hepes buffer,80 µl distilled water,
Substrate + Tracer Blind (no enzyme)	100 µl Reference substance (NN304 2 mg/ml dissolved in Hepes buffer), 20 -50 µl tracer (≈ 5 µCi/tube), 400 µl Hepes buffer, 80 µl distilled water.
Enzyme blind	100 µl Hepes buffer, 400 µl Hepes buffer, 80 µl *Staph. aureus* enzyme, 1,0 mg/ml.
Substrate	100 µl Reference substance (NN304 2 mg/ml dissolved in Hepes buffer), 400 µl Hepes buffer, 80 µl *Staph. Aureus* enzyme, 1,0 mg/ml.
Substrate + tracer:	100 µl Reference substance (NN304 2 mg/ml dissolved in Hepes buffer), 20 -50 µl tracer sample or tracer control (≈ 5 µCi/tube), 400 µl Hepes buffer, 80 µl Staph. Aureus enzyme, 1.0 mg/ml.

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After incubation for 3 hours at 37 °C in a heating cabinet the reaction was terminated by transferring the sample to a tube containing 277 mg guanidine hydrochloride (75 mm x 11.5 mm tubes). Then 60 μl 12.3 % (w/w) diammonium sulphate and 2.3 % (w/w) sulphuric acid were added to each tube. The reaction mixtures were transferred to HPLC vials and 100 μl of each sample were injected.

Chemistry [1-^{14}C]NN304

[1-^{14}C]Tetradecanoate N-hydroxysuccinimide ester(3): A solution of [1-^{14}C]myristic acid (0.18 mmol, 10 mCi, 1.67 mCi/ml) (1) in ethanol was evaporated to dryness using a flow of dry nitrogen. The residue was dissolved in acetone (5.0 ml). N-Hydroxysuccinimide (0,37 mmol, 42.49 mg) (2) was added. This mixture was then added to a solution of DCC (0.37 mmol, 75.72 mg in acetone (5 ml) over a 15 minutes period.

After 22 hours a TLC analysis showed 95% of (3) had been produced. The reaction mixture was filtered through a Whatman GF/B-filter and the filter was washed with acetone (2x2 ml). The combined filtrates were evaporated to dryness using a flow of nitrogen and the residue was dissolved in 5 ml acetone. The radiochemical yield was 9.4 mCi (94%). The crude (3) was used in the next step without any further purification.

Lys$^{\varepsilon B29}$-[1-^{14}C] tetradecanoyl ([^{14}C] NN 304) (5): 9.4 mCi (3) (0.17 mmol, 54 mg) in acetone was evaporated to dryness under a gentle flow of nitrogen. The residue was dissolved in N-methyl-2-pyrrolidone (NMP) (3 ml). Des(B30) human insulin (0.34 mmol, 1.92g) was dissolved in NMP (12 ml). Water (5 ml) and N-ethyldiisopropylamine (4 mmol, 640 μl) were added subsequently. The mixture became slightly warm. While stirring, (3) in NMP was added.

After 30 min at room temperature the radiochemical conversion was 56% as determined by HPLC system 1. After 90 min the NMP concentration in the reaction mixture was adjusted to 42% by the addition of water (15 ml). A greyish milky solution was formed. Ion-exchange purification of the crude product was performed on a semi-preparative scale.

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The pooled main fractions (200 ml) were prepared for C-18 SepPak by removing the ethanol from the eluent *in vacuo*. After evaporation the volume was adjusted to 300 ml by adding water (200 ml).

A 10 g Waters C-18 SepPak was activated with MeOH (2x10 ml) followed by washing with water (20 ml). The HPLC-eluate was eluted through the SepPak overnight. Subsequently, the SepPak was washed with water (2x10 ml), to remove any salts from the material.

The product was eluted from the SepPak using 40 ml of 0.1% TFA/acetonitrile (50/50) in 10 ml portions. Analysis by HPLC system 1 showed a RCP of < 97%. The radiochemical yield was 2.5 mCi (25%).

In order to obtain a RCP > 98% a reverse phase purification on a C-4 column was required. The above [^{14}C]NN304 solution was evaporated to dryness overnight in a Hetovac vacuum centrifuge. The residue was dissolved in 10 ml 0.1% TFA/acetonitrile (50/50). Purification was performed by HPLC using a C-4 column. In order to prevent further radiochemical degradation all fractions were kept cold during the entire purification.

The pH in the two fractions was adjusted to 7-8, using NaOH (2N). In the pH-range 4.5-7 the compound precipitated, but at pH > 7 the solutions remained clear. Each fraction was eluted through a Sep-Pak with only a minor loss of radioactivity (<5%). The product was eluted from the Sep-Pak with 70/30 EtOH/water containing 0.1M Tris and 0.05M phosphoric acid in portions of 5 ml. Fractions were pooled to give approximately 1.5 mCi [^{14}C]NN304 RCP > 98%. The overall radiochemical yield was 15%.

Conclusion

[^{125}I] TyrA14 NN304 and Lys$^{\epsilon B29}$ [1-^{14}C]- Tetradecanoyl des(B30) insulin were synthesised for ADME studies. The NN304 was radio-iodinated using the lactoperoxidase/hydrogen peroxide method. The [^{125}I] TyrA14 NN304 isomer was isolated using RP HPLC. Radio-peptide mapping confirmed the [^{125}I] labelling position of the isolated product as Tyrosine 14 in the A-chain of the NN304 molecule. The radiochemical yield was 30% with a radiochemical purity of

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>98%. [^{14}C]NN304 was prepared in two steps starting from [1-^{14}C] myristic acid and subsequently purified by HPLC. The overall radiochemical yield was 15% with a radiochemical purity >98%.

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