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The semisynthesis of [octadeutero-Phe^{B1}-octadeutero-Val^{B2}]-porcine insulin and its characterization by mass spectrometry

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Insulin analogues labelled with stable isotopes (e.g. deuterium, ^{18}O, ^{15}N, etc.) are authentic (the native structure is rigorously maintained), non-radioactive (preferred for injection into man) and can easily be distinguished from endogenous insulin by mass spectrometry by virtue of their molecular masses. Appropriate combinations of amino-protecting groups (methylsulphonylethyloxycarbonyl and *t*-butoxy carbonyl), Edman degradation and chemical coupling were used to produce [octadeutero-Phe^{B1}]-porcine insulin and [octadeutero-Phe^{B1}-octadeutero-Val^{B2}]-porcine insulin. The analogues were characterized by electrospray ionization mass spectrometry. Standard mixtures of labelled and unlabelled insulins were successfully studied by mass spectrometry. Isotope dilution mass spectrometry could therefore provide a useful direct measure of insulin under true physiological conditions, without many of the drawbacks of existing methods. In this regard, the analogue with 16 deuteriums was more suitable than the octadeuterated analogue, since the greater mass difference between the labelled and unlabelled forms enabled a lower mass spectrometric resolution to be used, resulting in higher sensitivity.

Key words: electrospray/insulin/mass spectrometry/quantitation/semisynthesis

Introduction

Studies of insulin metabolism in man have relied very heavily on the use of radioiodinated insulin (Hamlin and Arquilla, 1974; Stentz *et al.*, 1982). Such derivatives present the disadvantages, in addition to their radioactivity, that they may change the behaviour of the molecule under certain circumstances (Sodoyez *et al.*, 1973), although some authors (e.g. Freychet *et al.*, 1971; Glieman *et al.*, 1979) have found no loss in biological activity. Semisynthetic methods (Offord, 1980) have allowed the production of a variety of isotopically substituted insulin analogues which have been used for pharmacokinetic and mechanistic studies, mainly *in vitro* or *in vivo* in animals. These analogues, usually carrying a tritium label, have the advantage over iodinated insulin derivatives that their native structure and biological activities are rigorously maintained. Halban and Offord (1975) produced a semisynthetic tritiated insulin by specifically replacing Phe^{B1} with a tritiated phenylalanine. Davies and Offord (1985) synthesized tritiated insulin specifically labelled at Gly^{A1}. Specific labelling of other sites with ^{13}C (Saunders and Offord, 1972), deuterium (on Tyr^{A14}, Savoy *et al.*, 1988) or ^{18}O (on Lys^{B29}, Markussen *et al.*, 1983; Rose *et al.*, 1984) have also been achieved. Labelling with isotopes of oxygen and hydrogen has permitted, for example, the elucidation of the mechanistic process of trypsin-catalysed semisynthesis of human insulin by fast atom bombardment mass spectrometry (Rose *et al.*, 1991) or the measurement of steady-state turnover and metabolic clearance of insulin in dogs (Morishima *et al.*, 1985). The biosynthesis of specifically tritiated insulin could be achieved using *in vitro* cultures of rat pancreatic islet cells grown in a medium containing tritiated phenylalanine or isoleucine (Misbin *et al.*, 1981).

In order to pursue our pharmacokinetic studies in man, we have chosen to develop an analytical method based on isotope dilution mass spectrometry. To this end, we have synthesized and characterized two new analogues of porcine insulin and demonstrate that they can be used as internal standards for the quantitation of insulin.

Materials and methods

Reagents and solvents

Porcine insulin (monocomponent grade, zinc form) was purchased from Novo Industri, Denmark. Octadeutero-L-phenylalanine and octadeutero-L-valine (99 at.% deuterium, unlabile protons) were obtained from Cambridge Isotope Laboratories. Water was purified on a milli-Q system (Millipore). Dimethylformamide (DMF) and *N*-ethylmorpholine were purified as described by Halban and Offord (1975). Pyridine was redistilled at atmospheric pressure (b.p. 110°C). Phenylisothiocyanate (PITC, sequence analysis grade; Fluka) was redistilled under reduced pressure with a nitrogen bleed at 48–50°C. Urea was made up as an 8 M solution and passed through a column of Amberlite MB3 resin (BDH) immediately before use. Diethyl ether was dried over sodium metal and then passed through a glass column containing fresh, dry aluminium oxide 90 (active, neutral, 70–230 mesh ASTM, class I; Merck), the first two column volumes of solvent being discarded. All reactions took place in hermetically sealed tubes. All other reagents and solvents were of analytical grade or better, were obtained from commercial sources and were used without further purification unless otherwise stated.

Mass spectrometry

Mass spectrometric analyses of the title compound were carried out on a VG-Trio 2000 instrument fitted with a 3000 a.m.u. RF generator (VG Biotech, Manchester, UK). The mass spectrometer was equipped with an electrospray ion source (ESI-MS) and operated in positive ionisation mode under control of the Lab-Base data system. A mass spectrometric resolution of ~500 and a scan rate of 10 s from *m/z* 800 to 1500 were generally used. Data were processed using a six-point smoothing routine. Quantitative analyses of [octadeutero-Phe^{B1}]-insulin were carried out on a Bio-Q instrument (VG Biotech). Aliquots (10 μl) were injected at a flow rate of 5 μl/min. The quadrupole was scanned at 11 s/scan cycle from *m/z* 1140 to 1180 to cover the insulin peaks carrying five charges. A 13-point smoothing routine was

used to process the data. The quadrupole resolution was adjusted to approach as nearly as possible the theoretical resolution set by the isotope distribution, without sacrificing too much sensitivity. External calibration of the mass scale was performed with a solution of horse myoglobin (Sigma).

Reversed phase HPLC

Analytical HPLC was performed with the same equipment, column and solvents as described by Rose *et al.* (1991). For purification steps, HPLC was performed using equipment from Waters Associates consisting of two M-45 pumps, a model 720 system controller and a model 440 absorbance detector with a prior pass through an extended wavelength module (229 nm). The column was 250×10 mm diameter packed with Nucleosil C_{18} 7 μm 300 Å particles (Macherey Nagel) and operated at a flow rate of 4 ml/min. Solvent A was 100 ml 3 M $(NH_4)_2SO_4$, adjusted to an apparent pH of 2.7 (glass electrode) with concentrated sulphuric acid prior to making up to 1 l with water. Solvent B was 100 ml 3 M $(NH_4)_2SO_4$, adjusted to pH 2.7 with concentrated sulphuric acid prior to addition of 350 ml acetonitrile and making up to 1 l with water.

Preparation, purification and activation of t-butoxycarbonyl (BOC)-octadeutero-valine

Octadeutero-valine (16.07 mg, 150 μmol) was dissolved in 1 ml water, 1 ml DMF and 25 μl *N*-ethylmorpholine were added. The pH was found to be between 8 and 9 with paper pH indicator strips (0−14 range; Merck) previously moistened with water. Di-*t*-butyl dicarbonate (202.5 μl) was added at room temperature and the reaction was stirred for 20 h at room temperature. The reaction was followed by thin layer chromatography (TLC, silica 60), as described by Davies and Offord (1985) for BOC-glycine, and found to be complete on ninhydrin and HCl/ninhydrin staining. The product was vacuum-dried, resuspended in 1 ml 50 mM HCl and extracted six times with 2 ml ethyl acetate. The extraction efficiency was checked using the same TLC system. The six fractions of ethyl acetate were pooled, evaporated to dryness and the product stored at −20°C until required for activation.

The residue was then dissolved in 1 ml DMF and *N*-hydroxysuccinimide (20.4 mg, 100 μmol in 1 ml DMF) and *N,N'*-dicyclohexylcarbodiimide (DCC, 11.52 mg, 100 μmol in 1 ml DMF) were added to it. The reaction was left at room temperature for 5 h, with stirring every 30 min, and the formation of a white precipitate (dicyclohexyl urea) was observed. The tube was centrifuged for 2 min in a bench centrifuge and only the supernatant was used. The activation reaction was followed by TLC on silica gel (Kohn and Wilchek, 1982) using chloroform/methanol (9:1 v/v) as solvent (1 μl reaction mixture and 1 μl DCC at the same concentration were spotted on a TLC plate). After migration, the plate was sprayed with a solution containing 0.5 g *N,N*-dimethylbarbituric acid in 10 ml pyridine/water (9:1 v/v). No remaining DCC was detectable.

Preparation, purification and activation of methylsulphonylethyl-oxycarbonyl (MSC)-octadeutero-phenylalanine

Octadeutero-phenylalanine (100 mg) was slowly disolved in 3 ml dimethylsulphoxide (DMSO), and 100 μl *N*-ethylmorpholine were added to obtain a pH of 8.8. Methylsulphonylethyloxy-carbonyl-*N*-hydroxysuccinimide ester (350 mg, 2 Eq) was added and the solution was stirred at room temperature. The reaction was followed by electrophoresis on paper at pH 6.5, as described by Davies and Offord (1985), and found to be quantitative after 1 h. Acetic acid (100 μl) and 0.1% (w/v) trifluoroacetic acid (TFA) (50 ml) were added to the mixture and the product was purified on Sep-Pak-C_{18} cartridges (Waters Associates, Milford, MA, USA). After washing the cartridge with methanol (10 ml) and then 0.1% TFA (10 ml), 8 ml portions of the reaction mixture were loaded, 10 ml 0.1% TFA were used to wash the cartridge and the product was eluted in 4 ml 50% acetonitrile containing 0.1% TFA. After washing with 80% acetonitrile (10 ml) and re-equilibration of the cartridge with 0.1% TFA (10 ml), the loading and purification process was repeated seven times and the fractions of purified product were pooled, evaporated under a stream of nitrogen and freeze-dried. A white powder was obtained and stored at −20°C.

A part of the product (9.15 mg, 30 μmol) was dissolved in 500 μl DMF and *N*-hydroxysuccinimide (2.88 mg, 25 μmol, in 250 μl DMF) and DCC (5.16 mg, 25 μmol, in 250 μl DMF) were added. The activation reaction was left for 20 h at room temperature. Although no urea precipitated, the TLC test showed no trace of DCC in the reaction.

Preparation and purification of [des-Phe^{B1}-Val^{B2}, A1-B29-di-MSC]-porcine insulin

[A1-B29-di-MSC]-porcine insulin was prepared as previously described (Offord, 1980, p. 43), using 250 mg insulin as starting material. After 20 h of reaction under constant stirring, the product was precipitated with ice-cold dried diethyl ether (4 vol), washed three times with ether and carefully dried under reduced pressure. The product was analysed by electrophoresis on cellulose acetate, as previously described (Rose *et al.*, 1981), and purified by anion exchange chromatography on Sephadex A25, as described by Offord (1980, p. 44). The fractions of the main peak were pooled and desalted in seven portions on a Sep-Pak cartridge, washing with 10 ml 0.1% TFA and eluting with 4 ml 80% acetonitrile containing 0.1% TFA. The eluates were evaporated under a stream of nitrogen, pooled and freeze-dried overnight.

The product was then dissolved at 10 mg/ml in 8 M urea and purified stepwise by reversed-phase HPLC using the preparative system described above: 71% solvent B was found to be excellent for isocratic purification of the product. The acetonitrile present in the collection tubes was evaporated under a nitrogen flow and the product was desalted using a Sep-Pak cartridge, as above. A yield of 56.1 mg of pure [A1-B29-di-MSC]-porcine insulin was obtained. A sample was submitted for analysis on an automated amino acid sequence analyser (Applied Biosystems 477A).

A 25 mg/ml solution of the insulin derivative in pyridine/water (95:5 v/v) was prepared (2.25 ml) and 8.6 μl PITC were added (7.4 molar Eq). The reaction was left for 4 h at room temperature under constant stirring. The product was then precipitated and further washed three times with 8 ml dried ether, as previously described, and carefully dried under vacuum to give 50.1 mg of product. This was dissolved at 14 mg/ml in anhydrous TFA and left for 1 h at room temperature, being stirred for 15 s every 10 min. The volume was then reduced to less than 0.5 ml under a stream of filtered air. The protein was then precipitated with 8 ml ether, washed three times with 8 ml portions of ether and dried as above. A yield of 46.3 mg of [*des*-Phe^{B1}, A1-B29-di-MSC]-porcine insulin was obtained. A sample was purified by HPLC (analytical system) and examined by electrospray mass spectrometry.

The product (23 mg) was submitted to a second Edman degradation cycle, following the same procedure as described above, and 22 mg of dried [*des*-Phe^{B1}-Val^{B2}, A1-B29-di-MSC]-porcine insulin were obtained and purified by HPLC under

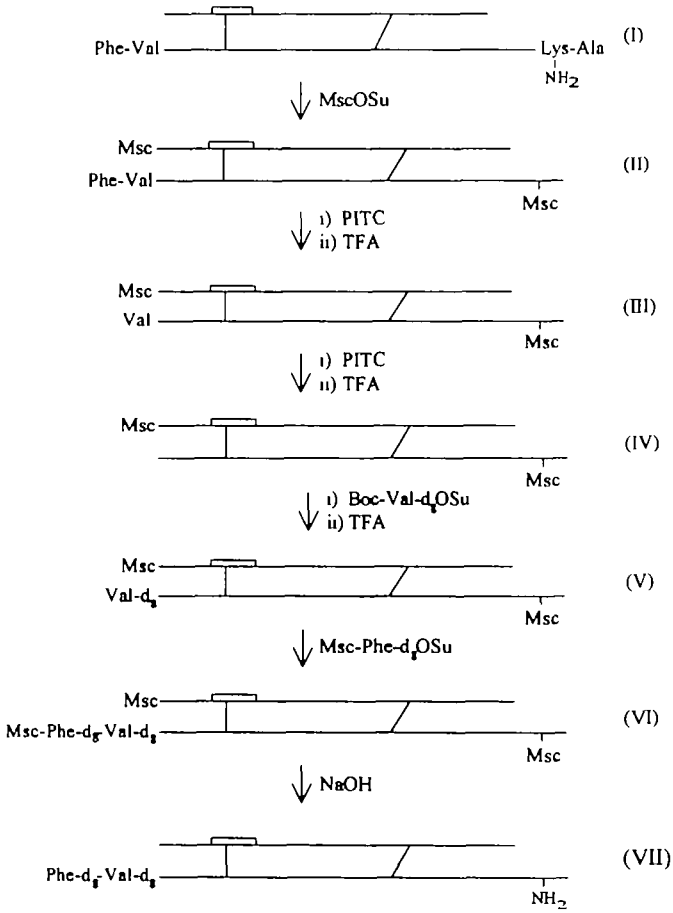

Fig. 1. Semisynthesis of [octadeutero-Phe^{B1}-octadeutero-Val^{B2}]-porcine insulin. (I) Porcine insulin; (II) [A1-B29-di-MSC]-insulin; (III) [*des*-Phe^{B1}, A1-B29-di-MSC]-insulin; (IV) [*des*-Phe^{B1}-Val^{B2}, A1-B29-di-MSC]-insulin; (V) [*des*-Phe^{B1}, octadeutero-Val^{B2}, A1-B29-di-MSC]-insulin; (VI) [MSC-octadeutero-Phe^{B1}-octadeutero-Val^{B2}, A1-B29-di-MSC]-insulin; (VII) [octadeutero-Phe^{B1}-octadeutero-Val^{B2}]-porcine insulin.

the preparative conditions as previously described. The product was examined by ESI-MS.

Synthesis and purification of [octadeutero-Phe^{B1}-octadeutero-Val^{B2}]-porcine insulin

To 8 mg of [*des*-Phe^{B1}-Val^{B2}, A1-B29-di-MSC]-porcine insulin, dissolved at 12 mg/ml in DMF, were added 2.7 ml freshly prepared BOC-octadeutero-Val hydroxy succinimide ester and 377 μl hydroxybenzotriazole (12 mg/ml) in DMF (0.67 mol hydroxybenzotriazole per mol active ester). *N*-ethylmorpholine (70 μl) was added to bring the apparent pH to a value between 8 and 9 as determined by paper pH indicator strips. The reaction was left for 21 h at room temperature and the pH was found still to be between 8 and 9. The product was then precipitated, washed three times with 8 ml portions of dried ether and vacuum-dried. The protein was then dissolved at 12 mg/ml in anhydrous TFA and left under stirring for 1 h at room temperature to remove the BOC protecting group on the octadeutero-valine in the B2 position. The volume was then reduced to less than 300 μl under a nitrogen flow and the insulin analogue precipitated and washed with ether, as above.

A portion of the residue (7.5 mg) was dissolved in 600 μl DMF and the preparation of 25 μmol activated MSC-octadeutero-phenylalanine added, followed by 166 μl hydroxybenzotriazole and 50 μl *N*-ethylmorpholine. The apparent pH was again between 8 and 9 and the reaction was set aside for 18 h at room temperature. The alkaline deprotection of the three MSC groups was carried out as described by Davies and Offord (1985) and the final product was purified on HPLC and examined by ESI-MS.

Synthesis and purification of [octadeutero-Phe^{B1}]-porcine insulin

A portion (1.2 mg) of the [*des*-Phe^{B1}, A1-B29-di-MSC]-porcine insulin was used in a coupling with activated octadeutero-Phe, similar to the one described above [bypassing steps (IV) and (V) of Figure 1]. The product was purified by HPLC and examined by ESI-MS.

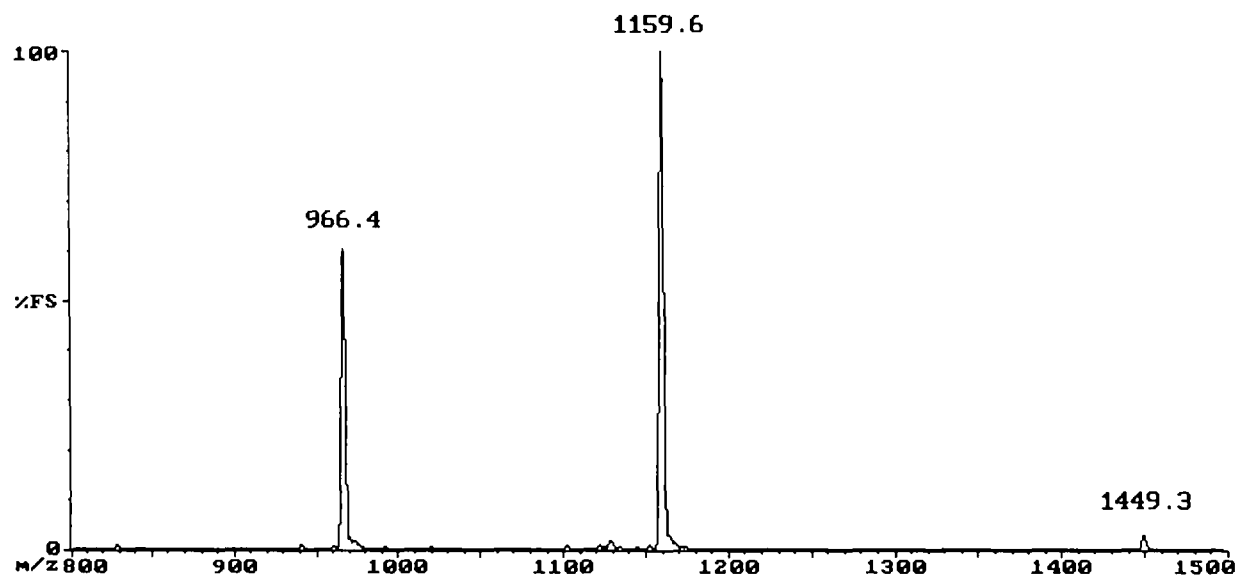

Fig. 2. Mass spectrum of the hexadecadeuterated analogue of porcine insulin (VII). [Octadeutero-Phe^{B1}-octadeutero-Val^{B2}]-porcine insulin. The measured molecular mass is 5792.8 and the calculated mass is 5793.7. The raw data have been processed using a 13-point smoothing routine. The mass spectrum shows three peaks on the mass to charge (*m*/*z*) scale, corresponding to the same molecule carrying different charges (z) due to 6, 5 and 4 protons.