Synthesis of [U-^{14}C]Arg labelled decapeptide cetrorelix, a novel lutenizing hormone - releasing hormone antagonist

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Abstract

The decapeptide cetrorelix is a novel luteinizing hormone - releasing hormone (LH-RH) antagonist. For nonclinical studies concerning absorption, distribution, metabolism and excretion (ADME) in animals the [^{14}C]-labelled compound is essential. Therefore, [U-^{14}C]Arg cetrorelix acetate salt was synthesized by Amersham International, Buckinghamshire (England) from precursor peptides provided by Degussa AG, Hanau-Wolfgang (Germany). [U-^{14}C]Arg labelled cetrorelix peptide base has a specific activity of 8.13 MBq/mg (220 µCi/mg) and a molecular weight of 1442.6 g/mol at this specific activity. The chemical and radiochemical purity (92.2 % decapeptide content and 97.6 %, respectively) determined by HPLC is suitable for nonclinical investigations.

Key words: Cetrorelix, radio synthesis, mass spectrometry, HPLC-analysis

1 INTRODUCTION

The decapeptide cetrorelix (Figure 1) was synthesized by the research group of Schally (1) and is currently under nonclinical and clinical development for oncological and gynaecological indications. Cetrorelix was characterized as a potent antagonist of LH-RH (Luteinizing Hormone-Releasing Hormone) (2). LH-RH antagonists have the potential for the treatment of hormone sensitive tumors like prostate (3, 4, 5) and mammary (6) carcinomas. In contrast to former antagonists, cetrorelix is devoid of side effects such as histamine release and induction of transient edema (4). In toxicological studies the safety of subcutaneously

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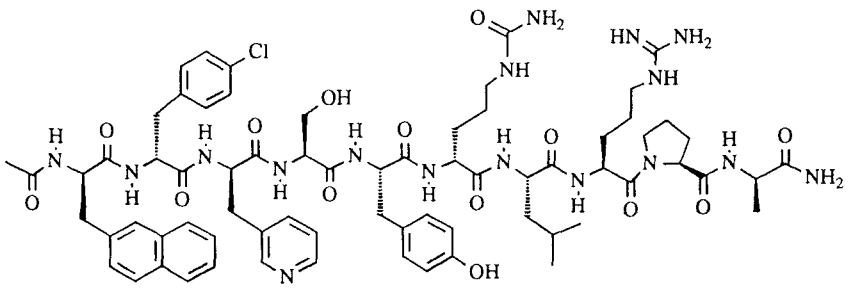
administered cetrorelix acetate salt has been confirmed. For development of cetrorelix acetate salt as a new pharmaceutical entity, investigations concerning absorption, distribution, metabolism and excretion (ADME) in animals are essential. This investigations are usually done with the [^{14}C]-labelled compound.

The nonphysiological amino acids within the peptide chain of cetrorelix acetate salt are responsible for a strong fluorescence activity of the molecule. This fluorescence can be assigned to the N-terminal part of the peptide. By labelling the C-terminus of the peptide with a [^{14}C]-labelled amino acid a double labelling of the molecule, with a flourescence label and a radio label, is achieved. This is of advantage for metabolism studies, because cleavage of the peptide chain can be realized by comparison of the HPLC chromatograms of these two labels. [U-^{14}C]Arg was chosen as radiolabel, because of its easy availability and its high specific activity which can be achieved with this uniformly [^{14}C]-labelled compound.

2 EXPERIMENTAL

2.1 Reagents and chemicals

The precursor peptides [Ac-D-Nal^1-(p-Cl)-D-Phe^2-D-Pal^3-Ser(^{t}Bu)^4-Tyr(^{t}Bu)^5-D-Cit^6-Leu^7] and [Pro^9-D-Ala^{10}-OMe x HCl] were provided by Degussa AG, Hanau-Wolfgang (Germany). Z^1-[U-^{14}C]Arg is a product of Amersham International, Buckinghamshire (England). All other chemicals were purchased in analytical grade from Aldrich, Dorset, (England).



Ac-D-Nal^1-(p-Cl)-D-Phe^2-D-Pal^3-Ser^4-Tyr^5-D-Cit^6-Leu^7-Arg^8-Pro^9-D-Ala^{10}-NH$_2$

Figure 1: Structural formula and amino acid sequence of cetrorelix

[1] Z = Carbobenzoxy

2.2 Preparation of [U-^{14}C]Arg labelled cetrorelix acetate salt

2.2.1 Preparation of Z-[U-^{14}C]Arg(HCl)-Pro-D-Ala-OMe

Z-[U-^{14}C]Arg (122.6 mCi (4.54 GBq), 0.39 mmol), H-Pro-D-Ala-OMe x HCl (150.9 mg, 0.638 mmol), dry pyridine (3.1 ml) and phosphorous trichloride (38 µl) were combined in a flask, stirred in an ice bath and allowed to warm up over 2 h. After stirring additionally at 80 °C for 4 h, the mixture was evaporated in vacuo, heated with 1-butanol at 70 °C for 0.5 h, evaporated and treated with isopropyl ether. The residue was dissolved in methanol (20 ml) to give Z-[U-^{14}C]Arg(HCl)-Pro-D-Ala-OMe and was analysed by TLC as 78 % RCP[2]

2.2.2 Preparation of Z-[U-^{14}C]Arg(HCl)-Pro-D-Ala-NH$_2$

Ammonia was bubbled through Z-[U-^{14}C]Arg(HCl)-Pro-D-Ala-OMe in an ice bath for 25 min and left at +2 °C overnight. The reaction mixture was evaporated in vacuo and the residue purified by HPLC. The main fraction was counted as 96.3 mCi (3.57 GBq) and analysed by TLC as 95 % RCP. The solution was evaporated in vacuo and the residue desiccated to give Z-[U-^{14}C]Arg(HCl)-Pro-D-Ala-NH$_2$ in a total amount of 215.1 mg.

2.2.3 Preparation of H-[U-^{14}C]Arg(HCl)-Pro-D-Ala-NH$_2$

Z-[U-^{14}C]Arg(HCl)-Pro-D-Ala-NH$_2$ in methanol (11 ml) with 10 % palladium on carbon (80 mg) was hydrogenated overnight. Additional catalyst (79 mg) was added and the hydrogenation continued for 2 days. The reaction mixture was filtered, evaporated in vacuo and washed with isopropyl ether. The residue was desiccated to give H-[U-^{14}C]Arg(HCl)-Pro-D-Ala-NH$_2$ in a total amount of 147.7 mg, ~90 % RCP as analysed by TLC.

2.2.4 Preparation of [U-^{14}C]Arg labelled cetrorelix

A suspension of H-[U-^{14}C]Arg^{8}(HCl)-Pro^{9}-D-Ala^{10}-NH$_2$, [Ac-D-Nal^{1}-(*p*-Cl)-D-Phe^{2}-D-Pal^{3}-Ser(^{t}Bu)^{4}-Tyr(^{t}Bu)^{5}-D-Cit^{6}-Leu^{7}] (498.6 mg, 0.402 mmol) and HOBt (72.1 mg, 0.53 mmol) in dry DMF (10 ml) was cooled in an ice bath, and DIC (67 µl, 0.43 mmol) was added. The mixture was allowed to warm up to room temperature, and stirred for 24 h. The mixture was poured into ethyl acetate and the precipitate filtered off, then dissolved in hot methanol and evaporated in vacuo to give [U-^{14}C]Arg cetrorelix .

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[2]RCP = Reactionproduct

2.2.5 Preparation of [U-^{14}C]Arg labelled cetrorelix acetate salt

[U-^{14}C]Arg cetrorelix was stirred in concentrated hydrochloric acid for 5 h and evaporated in vacuo. Water was added, evaporated in vacuo and the residue was purified by reversed phase HPLC using a buffer system of ammonium acetate / acetic acid to give [U-^{14}C]Arg cetrorelix acetate salt which was counted as 36.7 mCi (1.36 GBq) and analysed by TLC as 98.4 % and 98.7 %. The solution was freeze-dried to give a total amount of 180.3 mg peptide, which was counted as 36.37 mCi (1.35 GBq), resulting in a specific activity of 202 µCi/mg (7.46 MBq/mg). HPLC analysis gave 98.5 % RCP.

2.3 Chemical and radiochemical purity

The peptide content of [U-^{14}C]Arg cetrorelix acetate salt was determined via HPLC by comparing peak areas of equal amounts of the radiolabelled peptide with an unlabelled cetrorelix sample of known peptide content.

2.4 Solutions

For HPLC-analysis the following solutions were prepared:

Unlabelled standard:	Cetrorelix acetate salt in 0.01 mol/l acetic acid 1000 ng/ml
Radiolabelled peptide:	[U-^{14}C]Arg labelled cetrorelix acetate salt in 0.01 mol/l acetic acid 1000 ng/ml

2.5 HPLC equipment and chromatographic conditions

The HPLC equipment for the purity analysis consisted of a Hewlett-Packard 1090 M liquid chromatograph with a diode array detector, a Hewlett-Packard 1046 A fluorescence detector and the Hewlett-Packard HPLC Pascal workstation (Hewlett-Packard, Waldbronn, Germany). LiChrosphere 60 RP-Select B columns (250 x 4 mm 5 µm particle size) for the analysis of basic components were supplied by E. Merck (Darmstadt, Germany). The elution system consited of ammonium acetate buffer (0.05 mol/l, pH 4) as solvent A and a mixture of methanol - acetonitrile (1 : 1 / v : v) as solvent B. Elution was carried out isocratically with a mixture of 49 % ammonium acetate buffer (0.05 mol/l, pH 4) and 51 % methanol - acetonitrile (1 : 1 / v : v) at a flow rate of 0.4 ml / min. Cetrorelix was detected by fluorescence: excitation wavelength, 227 nm; emission wavelength, 340 nm (7).

3 Results and Discussion

3.1 Synthesis

The reaction was started with 122.6 mCi (0.39 mmol) Z-[U-^{14}C]Arg. As the result of five sythesis steps, a total amount of 180,3 mg or 0.116 mmol [U-^{14}C]Arg labelled cetrorelix acetate salt was available with a total radioactivity of 36.4 mCi (1.35 GBq). The synthesis was started with 122.6 mCi of Z-[U-^{14}C]Arg to produce Z-[U-^{14}C]Arg(HCl)-Pro-D-Ala-OMe. Therefore, the yield estimated on the basis of the radioactivity was 29.7 %. This result is in very good conformity with the chemical yield calculated by the total molar amounts which is also 29.7 %. The following figure gives a rough outline of the described synthesis

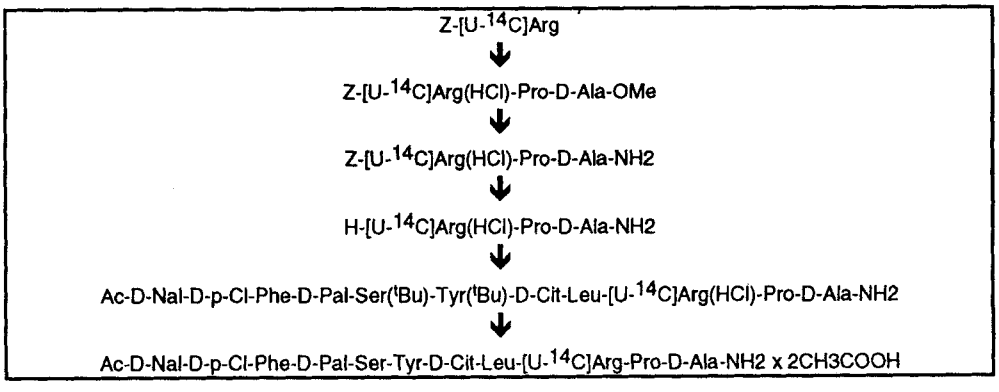

Figure 2: Reaction scheme of the synthesis of [U-^{14}C]Arg labeled cetrorelix acetate salt

The intermediates of the synthesis could be prepared in suitable purities (see method section) of about 78 to 95 %. The synthesis of [U-^{14}C]Arg labelled cetrorelix acetate salt resulted in a product of 98.5 % chemical purity.

3.2 Chromatography

The decapeptide cetrorelix exhibits a strong fluorescence activity. The excitation scan of cetrorelix shows a maximum at wavelength 227 nm, the emission scan has a broad maximum at 347 nm. The fluorescence detection is extremely sensitive, 2 ng/ml cetrorelix acetate salt can be determined quantitatively. To minimize the absorption of the peptide to glass vessel surface it is advantageous to solubilize the peptide salt in 0.01 mol/l acetic acid in which the peptide is stable over months (7).

3.3 Determination of chemical purity

For determination of the chemical purity [U-^{14}C]Arg labelled cetrorelix acetate salt was investigated by HPLC and mass spectrometry. The mass spectra (figure 3) is consistent with the structure. The molecular weight of the labelled compound shows a shift of 12 compared to the unlabelled compound (1430.6) indicating the six [^{14}C]-atoms of uniformly radiolabelled arginine.

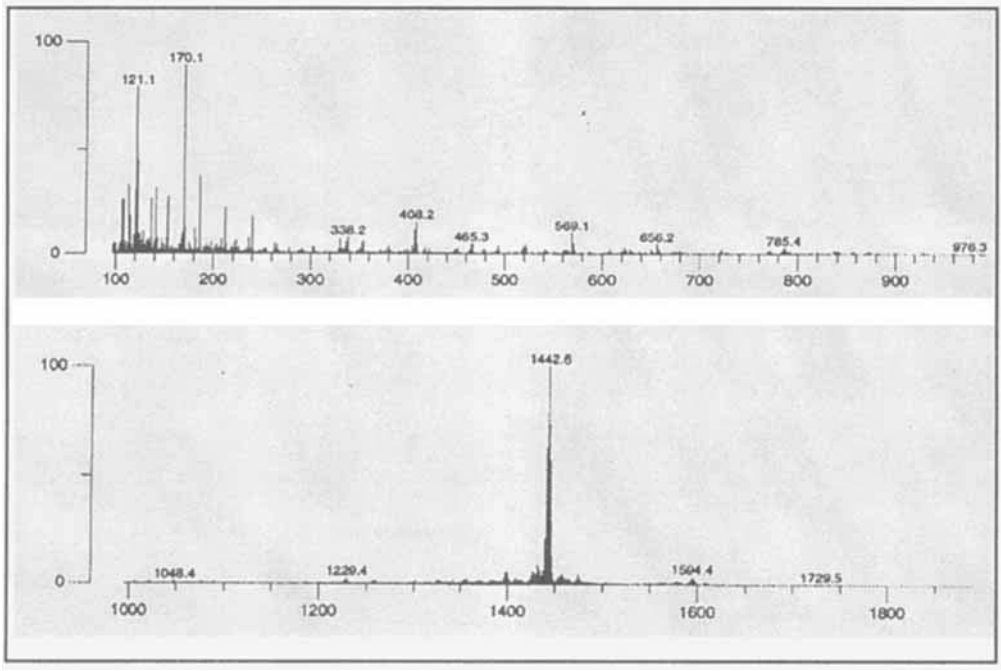

Figure 3: Mass spectra of [U-^{14}C]Arg labelled cetrorelix acetate salt

The peak areas of the [U-^{14}C]Arg labelled cetrorelix acetate salt and unlabelled cetrorelix acetate salt of known peptide content (ASTA Medica batch-No. FI 681) were compared by HPLC. Identical solutions of the radiolabelled and unlabelled peptide (1000 ng/ml) were prepared and analysed by HPLC (Table 1). 60 µl of each solution were injected. The unlabelled reference substance had a peptide content of 85.6 %. This results in a peptide content for [U-^{14}C]Arg labelled cetrorelix of 92.2 %.

Table 1: Chemical puritiy of [U-^{14}C]Arg labelled cetrorelix acetate salt as compared with unlabelled cetrorelix acetate salt (batch-No. FI 681)

Compound	Peak area	Peptide content (%)	nMol
Cetrorelix acetate salt	964	85.6	0.598
[U-Arg-^{14}C]cetrorelix acetate salt	1038	92.2	0.639

3.4 Determination of radiochemical purity

Radiolabelled solution (60 µl), containing 60 ng [U-^{14}C]Arg labelled cetrorelix acetate salt, was injected into the HPLC system. The peptide was detected by fluorescence, the fraction corresponding to cetrorelix was collected and radioactivity was measured by liquid scinitillation counting. The quench effect induced by the HPLC solvent has to be considered for calculation. For reference, 60 µl of the radiolabelled solution were directly measured. The calculated radiochemical purity was 97.6 % (table 2).

Table 2: Radiochemical puritiy of [U-^{14}C]Arg labelled cetrorelix acetate salt (n = 3)

[U-Arg-^{14}C]cetrorelix acetate salt		cpm
60 ng / 60 µl	Solution	843861
	(directly measured)	782551
		749025
	Mean:	**791812 = 100 %**
60 ng / 60 µl	HPLC-fraction	838264
	(quench corrected)	785166
		695827
	Mean:	**773086 = 97.63 %**

3.5 Specific activity of [U-^{14}C]Arg labelled cetrorelix peptide base

A total amount of 180.3 mg [U-^{14}C]Arg labelled cetrorelix acetate salt with a total radioactivity of 1.35 GBq (36.4 mCi) were synthesized. The peptid content of this batch was 92.2 %. Therefore, 166.2 mg of 180.3 mg [U-^{14}C]Arg cetrorelix acetate salt are [U-^{14}C]Arg labelled cetrorelix peptide base. Because of technical circumstances the specific activity of [U-^{14}C]Arg cetrorelix peptide base is of special interest for our

nonclinical investigations. The specific radioactivity of [U-Arg-^{14}C]cetrorelix peptide base (MW = 1442,6 g) is 11.7 GBq/mmol (314 mCi/mmol) or 8.13 MBq/mg (220 µCi/mg), respectively.

4 Conclusion

The radiolabelled decapeptide [U-^{14}C]Arg cetrorelix acetate salt was synthesized in high yield and high purity from its precursor peptides by incorporation of Z-[U-^{14}C]Arg. As shown by analytical data the radiolabelled decapeptide [U-^{14}C]Arg cetrorelix is free of any detectable amounts of undesired compounds. The specific radioactivity of [U-^{14}C]Arg cetrorelix base was calculated with 11.7 GBq/mmol (314 mCi/mmol). This specific radioactivity is sufficient for nonclinical experiments i.e. excretion balance, organ distribution and metabolism in laboratory animals.

5 References

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