SYNTHESIS OF *N,N*-DIDEMETHYLZOLPIDEM-*N*-{2-{3-(4-HYDROXY-3-[^{125}I] IODOPHENYL)}METHYL PROPIONATE} FOR USE IN RADIOIMMUNOASSAY

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Summary

The ^{125}I-derivative of zolpidem was prepared by iodination of a L-tyrosine methyl ester conjugate of a zolpidem precursor. The iodinated compound has been used as a tracer molecule in the development of a radioimmunoassay for zolpidem. It was purified by normal phase HPLC, in combination with gamma counting detection. The structure was confirmed by high resolution L-SIMS.

KEY WORDS = radiolabelled zolpidem - tyrosine methyl ester conjugate - 125-iodine - chloramine-T - radioimmunoassay

Introduction

A radioimmunoassay for the detection of zolpidem : *N,N*,6-trimethyl(4-methyl-phenyl)-2-imidazo[1,2-a]pyridine-3-acetamide, an imidazopyridine hypnotic (Stilnoct®, Stilnox®, Bikalm®, Ivadal®, Niotal®) [fig. 1] and its metabolites has been developed for the pre-screening of biological samples (1). Antibodies were produced in rabbits immunized with an immunogen, consisting of BSA coupled to 6-methyl-2-(4-methyl-phenyl)imidazo[1,2-a]pyridine-3-acetic acid. The latter compound was a precursor obtained during the synthesis of zolpidem.

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In this paper the preparation and purification of a ^{125}I-derivative of zolpidem is described, for its use as a tracer in the RIA procedure.

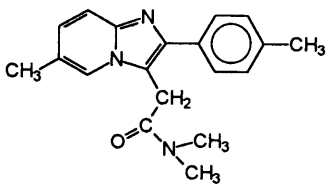

Fig. 1. Chemical structure of zolpidem

Many methods have been published for the synthesis of [^{125}I] labelled compounds (2). The chloramine-T method of Hunter and Greenwood (3,4) is the most widely used radioiodination technique. It has been widespreadly used for iodination of proteins, containing tyrosine residues, but also for small organic molecules with a phenolic group. Other compounds used for the iodination of small molecules are molecular iodine, obtained by oxidation of sodium iodide (5), iodine monochloride (6), enzymatic oxidation of iodide (7) and electrolytic production of "active iodine" (8). As zolpidem contains no phenolic group for direct iodination, the hapten was coupled to L-tyrosine methyl ester and subsequently labelled with ^{125}I by using the chloramine-T method [fig.2].

Experimental

Materials

6-methyl-2-(4-methyl-phenyl)imidazo[1,2-a]pyridine-3-acetic acid was synthesised in our laboratory by a slightly adapted procedure as described in the patent literature for the synthesis of zolpidem (9-11). It was also obtained as a gift from Synthélabo (France).

N,*N*-dimethylformamide (DMF) was obtained from UCB (Brussels, Belgium). *N*-hydroxy-succinimide (NHS) and *N*,*N'*-Dicyclohexylcarbodiimide (DCC) were ordered at Acros Chimica (Geel, Belgium). L-Tyrosine methyl ester was obtained from Fluka

Chemika-Biochemika (Buchs, Switzerland). Sodium metabisulphite, chloramine T (sodium toluene-p-sulphonchloramide trihydrate), sodium iodide, anhydrous sodium sulphate, methanol, dichlormethane, disodium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Merck (E. Merck, Darmstadt, Germany). Sodium [^{125}I]iodide IMS 30 (573.5 Mbq/μg of iodine) was obtained from Amersham International (Buckinghamshire, HP7 9NA, UK). Thin layer chromatography was done on Polygram Sil G/UV$_{254}$ plates (Machery-Nagel, Düren, Germany).

Equipment

High-performance liquid chromatography was carried out with a Merck Hitachi 6002 pump. Samples were injected into a Rheodyne injector (Model 7125)(Berkeley, CA, USA) supplied with a 200 µl sample loop. The analysis and purification of the reaction mixtures were performed on an analytical LiChrospher Si-60 5 µm column (125-4)(E. Merck). The column eluates were monitored with a Merck Hitachi L-3000 Photo Diode Array Detector (E. Merck) and with a gamma counter detector (Canberra Industries Inc., Connecticut, USA). The gamma counter detector consisted of a Bin/Power supply (Model 200), a 2 kV H.V. power supply (Model 3102D), a preamplifier/amplifier/discriminator (Model 814A) and a photo multiplier-tube base (Model 2007). Chromatograms were recorded with a Merck-Hitachi 2500 chromato-integrator. High resolution liquid surface-assisted secondary ion mass spectrometry (L-SIMS) was performed with a Kratos Concept 1 H instrument using a 6 keV Cs^+ beam and a thioglycerol matrix.

Methods and Results

A. Synthesis of *N,N*-didemethylzolpidem-*N*-{2-{3-(p-hydroxyphenyl)methyl propionate}}.

To a solution of 100 mg (0.36 mmol) 6-methyl-2-(4-methyl-phenyl)-imidazo[1,2-a]pyridine-3-acetic acid, dissolved in 9 ml DMF, 52 mg (0.45 mmol) *N*-hydroxy-

succinimide and 93 mg (0.45 mmol) *N,N'*-dicyclohexylcarbodiimide were added [fig. 2]. The mixture was stirred during 1 hour at room temperature and put in a refrigerator (4°C) overnight. The precipitate was removed by filtration and 58 mg (0.3 mmol) of L-tyrosine methyl ester was added to the filtrate. After mixing, the solution was allowed to stand at room temperature for 15 min. Progress of the reaction was monitored by TLC on silica plates with toluene-acetone-methanol (70:25:5,v/v) as mobile phase. The R_f values were as follows: hapten R_f = 0.15, NHS derivative R_f = 0.49, conjugation product R_f = 0.38. Purification was then carried out by column chromatography (silica gel 0.040-0.063 mm)(toluene-acetone-methanol). The yield was about 70 %. The conjugate was identified by high resolution liquid surface-assisted secondary ion mass-spectrometry (L-SIMS, positive ion mode, concept 1H). A 6 keV Cs^+ primary ion beam as impact energy, was applied to the purified reaction compound, dissolved in a thioglycerol matrix. The fragment ion at m/z 458,2086

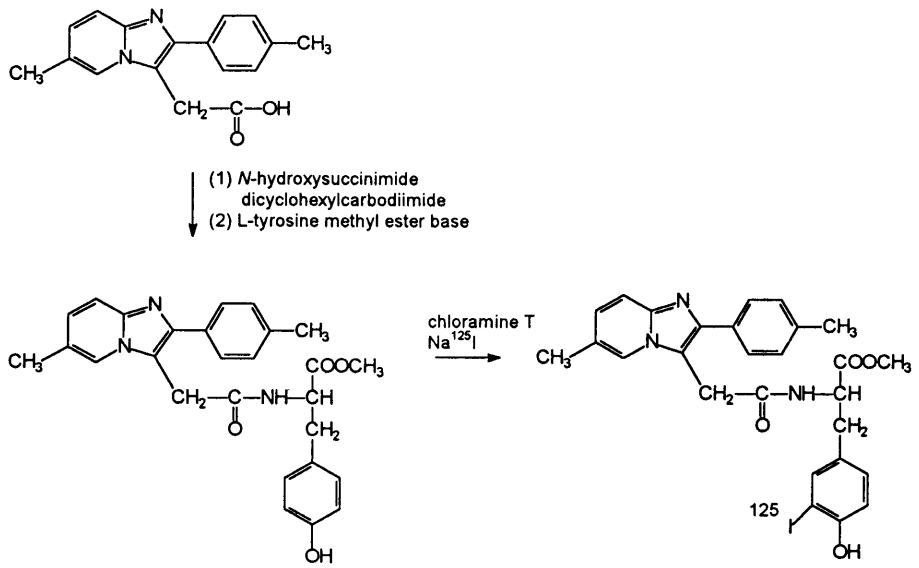

Fig. 2. Synthesis of *N,N*-didemethylzolpidem-*N*-{2-{3-(4-hydroxy-3-[^{125}I]-iodo-phenyl)}methyl propionate}

(calculated 458,2080) corresponds with the molecular ion +H ($C_{27}H_{28}N_3O_4$). The fragment ion at m/z 235,1214 (calculated 235,1235), corresponding with $C_{16}H_{15}N_2$, is formed by fragmentation of the parent molecule.

B. Synthesis of *N,N*-didemethylzolpidem-*N*-{2-{3-(4-hydroxy-3-[^{127}I]-iodophenyl)}-methyl propionate}

For the iodination of the L-tyrosine methyl ester conjugate of the hapten the oxidative chloramine-T method was used [fig. 2]. Several parameters were examined for the optimisation of the synthesis. All reactions took place in phosphate buffer pH 7.0 (0.066 M). Due to the low solubility of the conjugate in water, a minimum concentration of methanol was necessary. Experiments showed that 40 % of methanol was needed to keep all reaction products in solution.

a) Influence of temperature

The influence of the reaction temperature on the formation of the iodinated conjugate and side products was examined. A solution of 2.3 mg conjugate of L-tyrosine methyl ester with the hapten (5 μmol) in 5 ml MeOH was mixed with a solution of 1.6 mg NaI (10 μmol) in 6.5 ml of phosphate buffer pH 7 and kept at a temperature of respectively 0°C and 20°C. A solution of chloramine-T [1.4 mg (5 μmol)/200 μl phosphate buffer] was added and the mixture incubated for two minutes. The reaction was stopped with sodium metabisulphite [3.8 mg (20 μmol)/500 μl phosphate buffer], the mixture extracted with 25 ml of dichlormethane and further purified as described below. At 20°C, beside the monoiododerivative, the diiododerivative was also present. The latter product was not observed when the reaction temperature was 0°C.

b) Influence of chloramine-T concentration and reaction time.

The extent of iodination of the conjugate of L-tyrosine methyl ester with hapten was followed, using different concentrations of chloramine-T and during several time intervals. The amount of conjugate (10.9 nmol), NaI (2.5 nmol) and sodium metabisulphite (43.5 nmol) were kept constant. First the influence of increasing amounts of chloramine-T (3.75, 7.5, 11.25 and 15 nmol) was examined. The mixture was incubated for three minutes. The reaction mixtures were all examined with the HPLC procedure described below. The yield was estimated by measuring the peak areas of the conjugate of L-tyrosine methyl ester with the hapten and the iodinated conjugate assuming the same specific absorption for the two products calculated on a molecular weight basis. Yields obtained were respectively 3.8, 9.4, 11.2 and 11.3 %. Other oxidation products due to the higher concentrations of the oxidant were not observed. In another experiment the influence of the reaction time was studied. The reaction was performed with 11.25 nmol chloramine-T and stopped after respectively 3, 6 and 10 min. Yields obtained here were 11.2, 16.4 and 20.1 % respectively. Even higher yields were obtained when the oxidant was added in small portions during the reaction. The best results were obtained using three equal amounts (3.75 nmol) of chloramine-T, added at time intervals of 0, 3 and 6 minutes.The reaction was stopped after 10 min. The yield was 25.9 %.

c) Preparation and purification of ^{127}I-derivative

A solution of the L-tyrosine methyl ester conjugate of the hapten derivative (5 μmol/3.5 ml) in MeOH was mixed with 1.5 ml phosphate buffer pH 7.0 (0.066 M) and 5 ml of sodium [^{127}I] iodide solution (10 μmol/5 ml) was added. The temperature was kept at 0°C. Two hundred μl of a cold and freshly prepared chloramine-T solution (5 μmol/200 μl phosphate buffer) was added and the

mixture vortexed and kept at 0°C. After 3 and 6 minutes, another aliquot of 200 µl of chloramine-T was added. After 10 min, 500 µl of sodium metabisulphite (20 µmol/500 µl phosphate buffer) was added to neutralise the chloramine-T. The mixture was extracted with 25 ml dichlormethane. The organic layer was dried on anhydrous sodium sulphate, filtrated and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 1 ml mobile phase and 50 µl aliquots were injected into the chromatograph [fig. 3]. The mobile phase consisted of CH_2Cl_2-MeOH-acetonitrile (95:3:2, v/v), at a flow rate of 1 ml/min. The eluates were monitored at 320 nm. Repeated fractions of the iododerivative were collected and the mobile phase was evaporated to dryness under a stream of nitrogen. The yield of the labelling step was about 29 %. Mass spectrometric analysis (high resolution L-SIMS) of the molecule demonstrated the presence of the molecular ion ($C_{27}H_{26}N_3O_4I$) situated at m/z 584,1053 (calculated 584,1048)

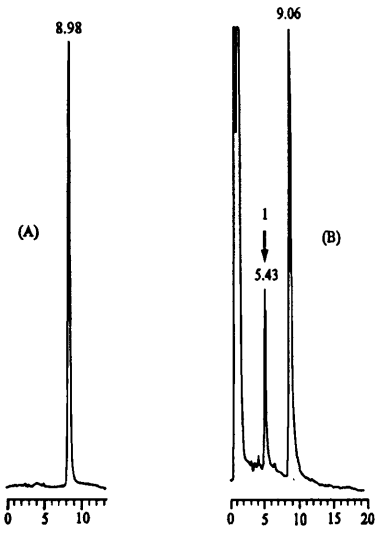

Fig. 3. HPLC chromatograms : from *N,N*-didemethylzolpidem-*N*-{2-{3-(p-hydroxy-phenyl)methyl propionate}} (A) and of the reaction mixture (B). (1 = *N,N*-didemethylzolpidem-*N*-{2-{3-(4-hydroxy-3-[^{127}I]-iodophenyl)}methyl propionate}

indicating the incorporation of one iodine. Major fragment ions are m/z 458,2071 (calculated for $[M+H]^+$ $C_{27}H_{28}N_3O_4$: 458,2080) and 235,1238 (calculated for $C_{16}H_{15}N_2$: 235,1235).

C. Synthesis of *N,N*-didemethylzolpidem-*N*-{2-{3-(4-hydroxy-3-[^{125}I]-iodophenyl)}-methyl propionate}

To a solution of 50 µl MeOH and 30 µl phosphate buffer, 10 µl (2.18 nmol) of a solution of the conjugate of the hapten with L-tyrosine methyl ester and 10 µl (1 mCi, 0.5 nmol) of a sodium [^{125}I]iodide solution were added. The temperature was kept at 0°C. Ten µl (0.75 nmol) of a freshly prepared chloramine-T solution were added and the reaction mixture vortexed. Another 10 µl aliquot of the chloramine-T solution was added at 3 and 6 minutes. After a reaction time of 10 min. Twenty µl of an aqueous solution of sodium metabisulphite (8.7 nmol) were added. The mixture was extracted with 300 µl dichlormethane and the organic layer dried on anhydrous sodium sulphate. The volume of the filtrate was adjusted to 950 µl with dichlormethane, followed by addition of 30 µl MeOH and 20 µl acetonitrile. One hundred µl aliquots were injected into the chromatograph. Gamma counting detection showed only one sharp peak, which had an excellent affinity for the antisera. No other radioactive compounds were detected. The radiochemical purity of the labelled compound is shown in fig 4. The specific radioactivity was assumed to be approximately that of iodine-125, i.e. about 2000 Ci/mmol. The fractions containing the iododerivative were collected, the mobile phase was evaporated under a stream of nitrogen and dissolved in phosphate buffer pH 7.4 (0.0667M) and stored at 4°C for use as a radio-tracer.

Fig. 4. HPLC chromatogram of purified tracer (gamma counting detection).

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