

**SYNTHESIS AND PURIFICATION OF DESMETHYLZOPICLONE-N-{3-(4-HYDROXY-3-[<sup>125</sup>I]-IODOPHENYL) PROPIONAMIDE}, A PROSTHETIC DERIVATIVE OF ZOPICLONE FOR USE IN IMMUNOASSAY.**

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**Summary**

Condensation of [<sup>125</sup>I]-Bolton/Hunter reagent with N-desmethylzopiclone lead to the title compound which was isolated and purified by normal phase HPLC, in combination with gamma counting detection. The introduction of the prosthetic group into the tracer precursor was proven by L-SIMS.

KEY WORDS = radiolabelled Zopiclone - [<sup>125</sup>I]-Bolton/Hunter reagent - radioimmunoassay

**Introduction**

A radioimmunoassay for the detection of Zopiclone : 6-(5-chloro-2-pyridyl)-6,7-dihydro-5H-pyrrolo[3,4-b]pyrazin-7-one 4-methyl-1-piperazine carboxylate, a cyclopyrrolone hypnotic (Imovane<sup>®</sup>, Zimovane<sup>®</sup>, Ximovan<sup>®</sup>, Amoban<sup>®</sup>, Cronus<sup>®</sup>) [fig. 1] and its metabolites has been developed for the pre-screening of biological samples (1). The antibodies have been raised following immunisation of rabbits with an immunogen, consisting of the

hemisuccinate derivative of N-desmethylzopiclone, coupled to BSA. This paper describes the preparation and purification of a  $^{125}\text{I}$ -derivative of the main metabolite, N-desmethylzopiclone, for use as a tracer in the RIA procedure. Many compounds do not have activated aromatic groups that lend themselves to direct radioiodination using chloramine T or another oxidant. One way to circumvent these problems is the use of prosthetic groups (2). The  $^{125}\text{I}$ -Bolton/Hunter reagent has been widely used as a iodination reagent (3), not only for proteins, but also for small organic molecules (4). This commercially available acylating reagent was therefore chosen for the preparation of radiolabelled N-desmethylzopiclone.

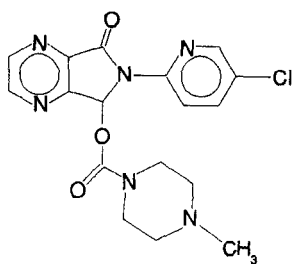


Fig. 1. Chemical structure of Zopiclone.

## Experimental

### Materials

N-desmethylzopiclone was synthesised in our laboratory by a slightly adapted procedure as described in the patent literature for the preparation of Zopiclone (5). It was also obtained as a gift from Rhône-Poulenc Rorer (France). 1,4-dioxane, methylene chloride, methanol, di-sodium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Merck (E. Merck Darmstadt, Germany). The  $^{125}\text{I}$ -Bolton/Hunter reagent (N-succinimidyl-3-(4-hydroxy-3- $^{125}\text{I}$ )iodophenyl)propionate), was purchased from NEN research products (Dupont de Nemours, Germany) and the Bolton/Hunter reagent (N-succinimidyl-3-(4-hydroxyphenyl)propionate) from Aldrich Chem. Co. (Belgium). Thin layer chromatography was done on Polygram Sil G/UV<sub>254</sub> plates (Machery-Nagel, Düren, Germany).

### Equipment

High performance liquid chromatography was carried out with a Merck Hitachi Model L-6002 pump, equipped with a Rheodyne injector (Model 7125)(Berkeley, CA, USA), supplied with a 200  $\mu$ l sample loop. The analysis and purification of the reaction mixtures were performed on an analytical LiChrospher Si-60 5  $\mu$ m 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. Liquid surface-assisted secondary ion mass spectrometry (L-SIMS) was performed with a Kratos Concept 1 H instrument using a 6 keV Cs<sup>+</sup> beam and a thioglycerol matrix.

### **Results and discussion.**

#### *A. Synthesis, purification and identification of desmethylzopiclone-N- $\beta$ -(4-hydroxyphenyl)-propionamide.*

1 mg (2.7  $\mu$ mol) N-desmethylzopiclone and 2 mg (7.6  $\mu$ mol) N-succinimidyl-3-(4-hydroxyphenyl)propionate (Bolton/Hunter reagent) were dissolved in 50  $\mu$ l dioxane and kept at room temperature [fig. 2.A.]. The reaction was evaluated by TLC on silica plates with CH<sub>2</sub>Cl<sub>2</sub> - MeOH (92.5 : 7.5, v/v) as mobile phase. After 6 hours, the starting material (N-desmethylzopiclone, R<sub>f</sub> = 0.08, UV 365 nm) had completely disappeared and the reaction product (R<sub>f</sub> = 0.25, UV 365 nm) was formed. Then the reaction mixture was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 1 ml mobile phase. 50  $\mu$ l aliquots were injected into the chromatograph. The mobile phase consisted of CH<sub>2</sub>Cl<sub>2</sub> - CH<sub>3</sub>CN - MeOH (91:6:3, v/v), at a flow rate of 1 ml/min. The eluates were monitored at 305 nm and showed only one major reaction product [fig. 3C]. This peak was collected and the mobile phase evaporated to dryness under a stream of nitrogen. Liquid surface-assisted secondary ion mass spectrometry (L-SIMS, positive ion mode, concept 1

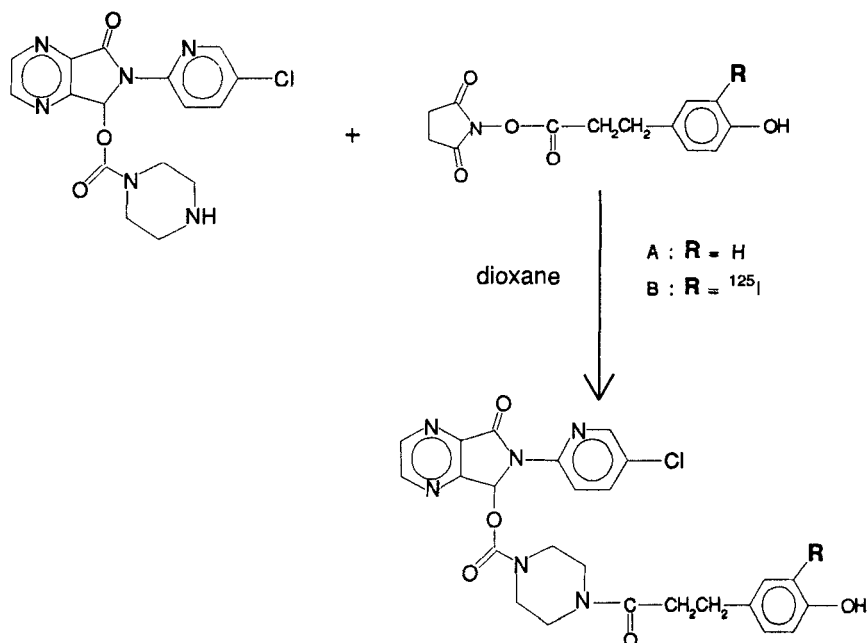


Fig. 2.A. Synthesis of desmethylzopiclone-N-{3-(4-hydroxyphenyl)-propionamide}.

Fig. 2.B. Synthesis of desmethylzopiclone-N-{3-(4-hydroxy-3-[<sup>125</sup>I]iodophenyl)propionamide}

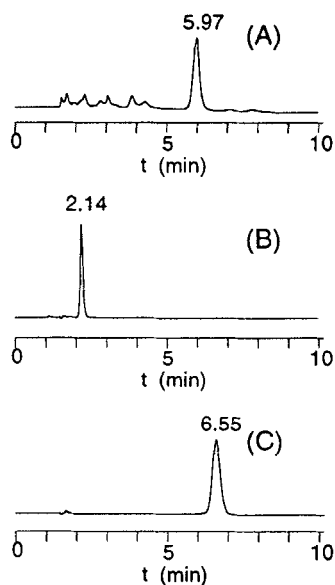


Fig. 3. HPLC chromatograms : N-desmethylzopiclone (UV 305 nm) (A), Bolton/Hunter reagent (UV 275 nm) (B), and the reaction mixture (UV 305 nm) (C).

H), using a 6 keV Cs<sup>+</sup> primary ion beam as impact energy, was applied to the purified reaction compound, dissolved in a thioglycerol matrix. The fragment ion at *m/z* 523 corresponds with the molecular ion +1, indicating the incorporation of the prosthetic group. The fragment ions at *m/z* 245 (244 +1) and *m/z* 279 (278 +1) are formed by fragmentation of the parent molecule as indicated in fig. 4.

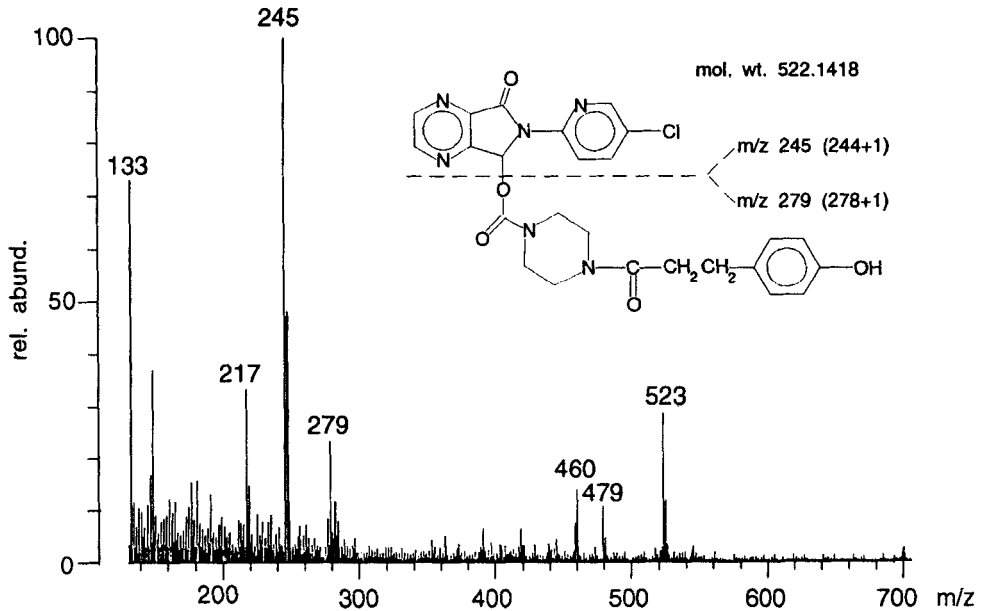


Fig. 4. Mass spectrum from desmethylzopiclone-N-{3-(4-hydroxyphenyl)-propionamide}

*B. Synthesis and purification of desmethylzopiclone-N-{3-(4-hydroxy-3-[<sup>125</sup>I]iodophenyl)-propionamide}.*

A solution of N-succinimidyl-3-(4-hydroxy-3-[<sup>125</sup>I]iodophenyl)propionate in anhydrous benzene (1 mCi, 100  $\mu$ l), with a specific activity of 2200 Ci/mmol, was carefully evaporated to dryness in the shipping vial under a stream of nitrogen. A solution of N-desmethylzopiclone in dioxane (50  $\mu$ l, 100  $\mu$ g/ml) was added to the above residue, the contents were mixed vigorously and left overnight at room temperature [fig. 2.B.]. 1 ml of mobile phase, consisting of CH<sub>2</sub>Cl<sub>2</sub> - CH<sub>3</sub>CN - MeOH (94:3:3, v/v), was added to the reaction mixture and 50  $\mu$ l aliquots were injected in the HPLC system. Gamma counting detection allowed the isolation of three main peaks of radioactivity [fig. 5C]. Peak n<sup>o</sup> 1 corresponded with the unreacted [<sup>125</sup>I]-Bolton/Hunter-reagent, whereas peak n<sup>o</sup> 2 remained unidentified and did

not show any affinity for the antiserum. Peak n° 3 showed an excellent affinity for the antiserum (1) and was most probable to correspond with the radioiodinated derivative of zopiclone. The radiochemical purity of the labelled compound is showed in fig. 5D. The isolated and purified tracer was evaporated to dryness, dissolved in phosphate buffer pH 7.4 (0.0667 M) and stored at 4 °C for use as a radio tracer.

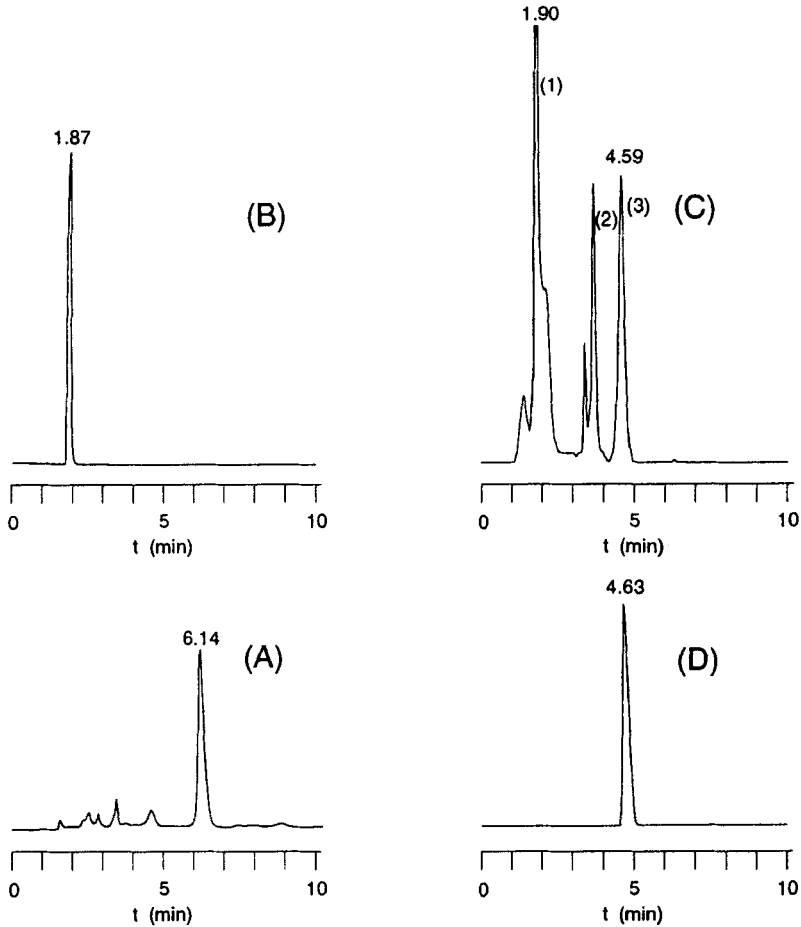


Fig. 5. HPLC chromatograms : N-desmethylzopiclone (UV 305 nm) (A) ; [<sup>125</sup>I]-Bolton/Hunter reagent (B), reaction mixture (C) and purified tracer (D) (gamma counting detection).

## **Conclusions.**

The Bolton/Hunter reagent was successfully used for the incorporation of a prosthetic group on the secondary amine function of N-desmethylzopiclone as could be confirmed by mass spectrometry (L-SIMS). Using a similar procedure, the commercially available [<sup>125</sup>I]-Bolton/Hunter reagent permitted to prepare the radiolabelled tracer. Normal phase HPLC, combined with gamma counting detection was shown to be a suitable technique for the isolation and purification of the synthesised tracer.

## **References**

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