Preparation and Purification of 7-Iodoclonazepam for use in Radioimmunoassay

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SUMMARY

A method is described for the preparation and purification of 7-[^{125}I]-Iodoclonazepam (5-(o-Chlorophenyl)-2,3-Dihydro-7-[^{125}I]-Iodo-1H-1,4-Benzodiazepin-2-one). The structure was confirmed by mass spectrometry using 7-[^{127}I]iodoclonazepam prepared by the same method. 7-[^{125}I]-Iodoclonazepam binds well to a benzodiazepine antiserum. Although readily displaced by all the benzodiazepines commercially available in the UK, it is not displaced by structurally related nonbenzodiazepines except at very high concentrations. 7-[^{125}I]Iodoclonazepam should therefore be useful for the development of a screening radioimmunoassay (RIA) for benzodiazepines.

KEYWORDS: BENZODIAZEPINE RADIOIMMUNOASSAY RADIOTRACER IODOCLONAZEPAM

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INTRODUCTION

There are two commercial kits currently available for use in forensic toxicology for the detection of benzodiazepines in biological fluids (Syva Emit DAU and Syva Emit TOX). The Emit DAU is specifically designed for use with urine samples and the Emit TOX for use with serum. The antisera in each kit are broadly specific to the benzodiazepine group of drugs and active metabolites; breakdown of the benzodiazepine structure to the benzophenone causes a substantial drop in cross-reactivity. In addition, there are a number of radioimmunoassays for the detection of specific benzodiazepines (1, 2, 3) and one screening assay using a tritiated label (4). The development of a general screening assay based on an ^{125}I labelled benzodiazepine would prove advantageous for several reasons; for example the Emit assays are not always appropriate for use with samples of forensic interest, specific RIAs are not useful for screening for groups of drugs, and assays based on tritiated labels are often complicated and time-consuming.

Radioiodinations are generally performed using a simple chemical method such as the chloramine-T reaction developed by Hunter and Greenwood (5). Although the Sandmeyer reaction has been used extensively for the addition of halogens to a benzene nucleus it has been used only rarely for labelling compounds with [^{125}I]-iodine. The reaction requires the presence of an aromatic amine which can then be converted to a diazonium salt with the diazo group finally being displaced by the required halogen. Benzodiazepines containing aromatic nitro groups were chosen as

starting materials for the synthesis described in this work as they could be easily reduced to the amine and then iodinated by a 'Sandmeyer type' reaction. It was anticipated that an iodinated benzodiazepine would be bound by either of the commercially available broadly-specific antisera.

EXPERIMENTAL

Materials

Sodium ^{125}-Iodide (614MBq/μg) was obtained from Amersham International plc, Amersham, Bucks, and trifluoroacetic acid from Aldrich Chemical Co., Gillingham, Dorset. All other chemicals were of Analar grade and were obtained from BDH Chemicals Ltd, Poole, Dorset.

Equipment

Thin-layer chromatography was carried out using pre-coated silica plates with fluorescent indicator (E. Merck, Darmstadt, FRG). Radioactive spots were located with a type 5.40 scintillation meter (Alrad Instruments Ltd, Newbury, Berks) modified by fitting a lead shield 3mm thick, in the centre of which was a small window (2 x 4mm), to improve resolution.

Gamma-counting was carried out using a NE 1600 counter (Nuclear Enterprises Ltd, Beenham, Berks) which had an efficiency of approximately 61% for [^{129}I]-iodine.

Low resolution mass spectra were obtained using a VG-Micromass 16F mass spectrometer (VG Micromass, Altrincham, Cheshire). The following conditions were used: emission, 100μA; electron energy 70eV; source temperature, 200°C. Data were collected using a VG 2250 Data System with the mass spectrometer scanning at 3 secs per decade.

METHODS AND RESULTS

Figure 1 shows the structures of the compounds in the synthetic route from clonazepam (I) to 7-iodoclonazepam (IV).

i) Preparation of 7-Aminoclonazepam (II)

Clonazepam (I; 40mg) was added to a mixture of hydrochloric acid (1M; 2ml) and tin granules (500mg) and the solution stirred for one hour at room temperature. The mixture was diluted with distilled water (8ml), basified with sodium hydroxide solution (1M) and then extracted with dichloromethane (2 x 8ml). The organic layer was dried with anhydrous sodium sulphate, and the solvent removed on a rotary evaporator. The yellow crystalline product (II), yield approximately 90%, gave only one spot (R_F 0.13) by TLC (silica; chloroform-acetone, 4 + 1). Clonazepam has an R_F value of 0.32 on this system.

ii) Preparation of 7-Pyrrolidine-triazine-clonazepam (III)

7-Aminoclonazepam (II; 22mg) was suspended in ice cold water (100µl), and sodium nitrite (5.5mg) in ice cold water (100µl) was then added. The temperature was kept below 5°C. Trifluoroacetic acid (16µl) was added slowly with stirring and, after a further 5 minutes stirring, an ice cold solution of pyrrolidine (1.5mg) in sodium hydroxide solution (1M; 36µl) was added; the product (III) precipitated immediately. The product was filtered, washed with ice cold water (1ml) and dried in vacuo over silica gel (yield approximately 80%). Only one spot (R_F 0.26) was observed by TLC (silica; chloroform-acetone, 4 + 1).

iii) Preparation and Purification of 7-[^{127}I]-Iodoclonazepam (IV)

The triazine (III; 10mg) was dissolved in dry acetonitrile (1ml) and an excess of potassium iodide (20mg) was added. The mixture was cooled to less than 5°C on an ice bath.

Trifluoroacetic acid (8.6µl) was added slowly while keeping the temperature below 5°C and the mixture was then allowed to rise to room temperature (approximately 2 hrs).

The reaction mixture was diluted with distilled water (2ml) and then extracted with dichloromethane (2 x 3ml). The organic layer was dried with anhydrous sodium sulphate and the solvent removed on a rotary evaporator. The product (IV; yield 80%) gave only one spot (R_F 0.36) by TLC (silica; chloroform-acetone, 4 + 1).

The product was identified by mass spectroscopy; the low resolution mass spectrum of 7-[^{127}I]-iodoclonazepam is shown in Figure 2. The molecular ion is observed at m/z 397, with major fragment ions at 396 (loss of H), 368 (loss of HCO), 361 (loss of HCl) and 333 (loss of HCOCl); the additional ions at m/z 269, 241 and 234 probably result from loss of ^{127}I from the ions at 396, 368 and 361 respectively.

iv) Preparation and Purification of 7-[^{125}I]-iodoclonazepam (IV)

A solution of the triazine (100µl, 1mg/ml) was added to a solution of trifluoroacetic acid in acetonitrile (100µl, 0.86µl/ml). This solution was cooled to 0°C and then a solution of sodium ^{125}-iodide (10µl, 37MBq) was added, and the mixture allowed to come to room temperature over a 2 hr period. The reaction mixture was diluted with a solution of sodium metabisulphite in 0.1M sodium hydroxide solution (1ml, 1mg/ml) and then extracted with dichloromethane (2 x 3ml). The organic extracts were combined, blown down under nitrogen and reconstituted with ethanol (20µl). Purification of the product was necessary due to the large excess of drug derivative used in the reaction. A simple TLC system (silica; chloroform-acetone,

4 + 1) was used to separate the 7-[^{125}I]-iodoclonazepam (R_F 0.36) from unreacted ^{125}-iodide (R_F 0.0), unreacted triazine (R_F 0.26; minor), and by-products of the reaction.

The 7-[^{125}I]-iodoclonazepam was removed from the plate by scraping off the appropriate band of silica and eluting with methanol. The product has proved to be chemically stable when stored at 4°C in methanol. Approximately 20% of the available ^{125}I is incorporated into the benzodiazepine derivative.

The specific activity of the purified tracer was determined by the method of Morris (6) and found to be 5.6TBq/mmol (12.7MBq/μg).

DISCUSSION

Iodination of a benzodiazepine, nitrazepam, via a triazine derivative has been described by Foster, et al. (7). The radiochemical yield for the reaction was only 10% and there were several by-products. The conditions described in the present work have been optimised for maximum radioiodine incorporation by using a large excess of triazine. This increases radiochemical yields to 20% and over, while also giving far fewer by-products than when the reaction is carried out under aqueous conditions.

The 7-pyrrolidine-triazine-clonazepam is easily purified and appears to be very stable. A stock of the compound can be prepared and the final iodination step (see (iv) above) performed as required. The iodination and the separation of reaction products by TLC are fairly quick and very easy to perform, giving a radiotracer of relatively high specific activity. The reaction and extraction stages take place in a single sealed tube, minimising the risk of contamination.

The radiolabelled product has been used for the development of a cheap, simple and effective screening assay for detection of

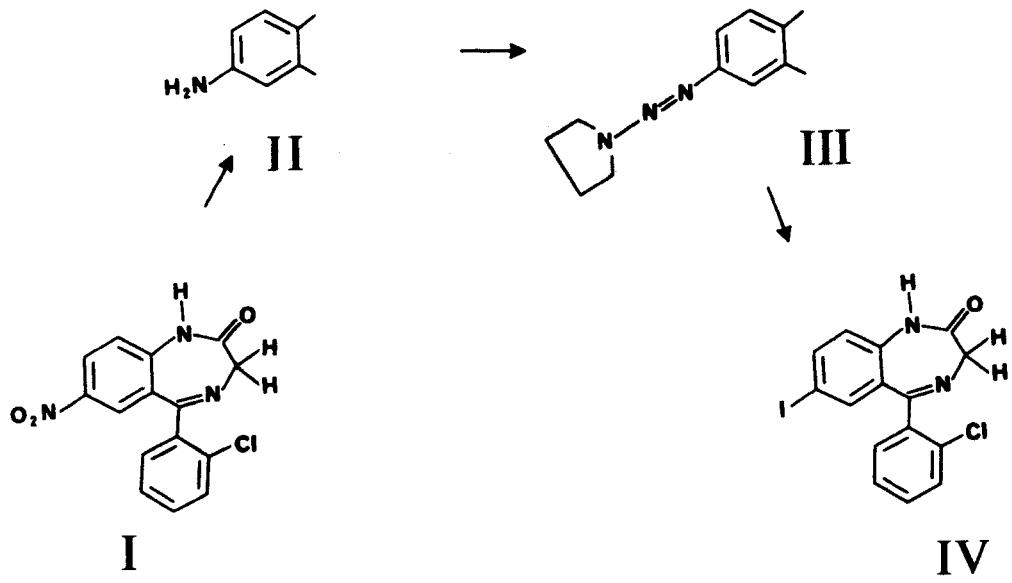

Figure 1. Structure of clonazepam (I) and synthesis of 7-aminoclonazepam (II), 7-pyrrolidine-triazine-clonazepam (III), and 7-iodoclonazepam (IV).

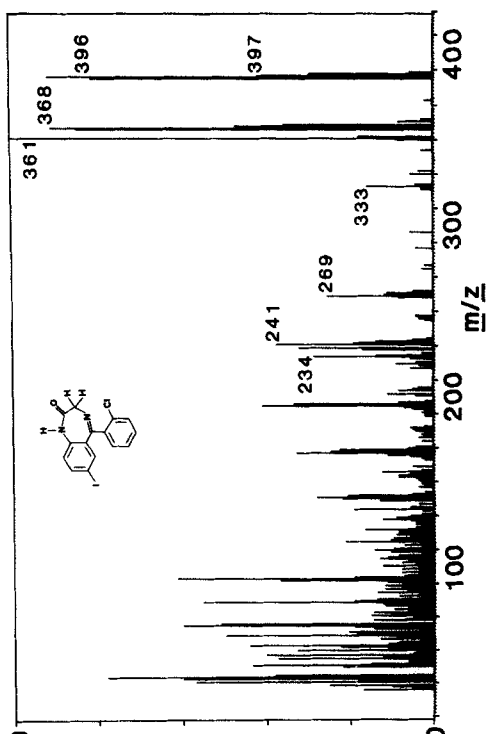

Figure 2. Mass spectrum and molecular structure of 7-[^{127}I]-iodoclonazepam.

benzodiazepines in biological samples submitted for forensic analysis (8).

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