# SYNTHESIS OF IODOBUPRENORPHINE FOR USE IN RADIOIMMUNOASSAY

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## Summary

The synthesis of [$^{125}$I]iodobuprenorphine is described. This compound has been used as a tracer molecule in the development of a new radioimmunoassay for buprenorphine. The parameters that effect the direct iodination of buprenorphine using sodium [$^{127}$I]iodide, in the presence of either chloramine-T or Iodo-Beads®, were previously studied. The structure of iodobuprenorphine was confirmed by mass spectrometry.

KEY WORDS = 125-iodine - iodobuprenorphine - chloramine-T - Iodo-beads® - radioimmunoassay.

## Introduction

A new radioimmunoassay for the detection of buprenorphine or [5α,7α(S)]-17(cyclopropylmethyl)-α-(1,1-dimethylethyl)-4,5-epoxy-18,19-dihydro-3-hydroxy-6-methoxy-α-methyl-6,14-etheno-morphinan-7-methanol [Fig. 1] in urine samples has been

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developed (1). The antibodies have been prepared by coupling the 2-diazo benzoic acid derivative of buprenorphine to BSA and by subsequent injection of the conjugate into rabbits. [$^{125}$I]iodobuprenorphine has been selected as a tracer for the development of an immunoassay that will be used in our laboratory for pre-screening urine samples. In various urine samples from drug users, HPLC analysis of buprenorphine and its major metabolite has clearly demonstrated in some cases the misuse of this drug (2).

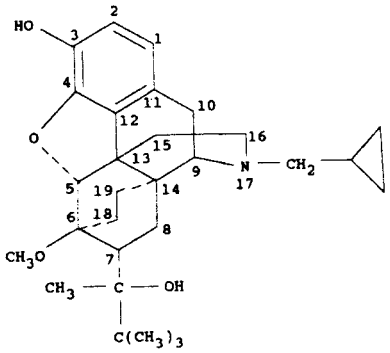


Fig. 1. Chemical structure of buprenorphine

Several oxidative iodination techniques have been described for the synthesis of [$^{125}$I] labelled compounds. The chloramine-T method, devised by Hunter and Greenwood (3, 4), has been widespreadly used for the incorporation of iodine into proteins, containing tyrosine residues, or into other compounds with a phenolic group. Other chemical oxidants have been proposed, such as 1,3,4,6 tetrachloro-3α,6α-diphenylglycoluril (5), iodine monochloride (6) and sodium hypochlorite (7), together with the enzymatic oxidation of iodide (8) and the electrolytic production of "active iodine" (9). As buprenorphine contains a phenolic group it was expected that iodination would be possible using the chloramine-T method, as shown for morphine (10).

The [$^{127}$I]iodobuprenorphine derivative was synthesised in order to determine its chemical characteristics and to optimise the reaction conditions and its purification. These methods have been applied to the synthesis of the radiolabelled compound.

## Experimental

### Materials

Buprenorphine.HCl was obtained from Reckitt and Colman (Pharmaceutical Division, Hull, U.K.). Sodium metabisulphite, chloramine-T (sodium toluene-p-sulphonchloramide trihydrate), potassium iodide, anhydrous sodium sulphate, methanol, chloroform, dichloromethane and n-hexane were purchased from Merck (E. Merck, Darmstadt, Germany). Dichloromethane p.a. was distilled prior to use. Sodium [$^{125}$I]iodide IMS 30 (606.8 MBq/μg of iodine) was obtained from Amersham International (Buckinghamshire, England HP7 9 NA).

As an alternative for chloramine-T, Iodo-beads® (Pierce, Rockford, Illinois, USA), were tried out as a iodination reagent. A Bond Elut C18 extraction cartridge (Analytichem International; Harbor City, CA, USA) was used for the separation of unreacted iodide and iodinated buprenorphine.

### Equipment

High-performance liquid chromatography was carried out with a Merck Hitachi 6002 pump. Samples were injected into a Rheodyne injector (Berkeley, CA, USA) supplied with a 200 μl injector valve. For the purification and identification of [$^{127}$I]iodobuprenorphine on a semi-preparative scale using the chloramine T method, the mobile phase was pumped through a stainless steel column (25 cm x 1.5 cm), packed with 15 g Lichrosorb 10

μm (E. Merck). The analysis of the reaction mixture, obtained with the Iodo-beads® method, was performed on a analytical LiChrosorb Si-60 5 μm column (25 x 0.4 cm I.D.)(E. Merck). The column eluate was monitored at 280 nm with an ultraviolet detector (Model 440, Waters Ass. Inc., Milford, USA).

A LiChrospher CN, 5 μm (25 cm x 0.4 cm I.D.) column, combined with an electrochemical detector (Model Eldec 201, Chromatofield/Instrulab, Chateauneuf-les-Martigues, France) and a gamma counter detector (Canberra Industries Inc., Connecticut, USA) was used to construct a calibration graph for calculation of the specific activity of [$^{125}$I]iodobuprenorphine and to monitor its purity. 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 photomultiplier-tube base (Model 2007).

Chromatograms were recorded with a Merck-Hitachi 2500 chromato-integrator. Radioactivity of the isolated HPLC fractions was counted on a gamma counter (Berthold BF 5300, Wildbad, Germany). The structure of iodobuprenorphine was confirmed by mass spectrometry (HP 5995A, Hewlett Packard, USA).

## Methods and Results

### A. Synthesis of [$^{127}$I]iodobuprenorphine

Several parameters were examined for the optimisation of the synthesis of [$^{127}$I]iodobuprenorphine. The oxidative chloramine-T method was applied. All reactions were performed in phosphate buffer pH 7.0 (0.066 M).

a) Influence of solvent composition.

When working in aqueous conditions, only 1 % iododerivative

was formed, due to solubility problems. Different experiments showed that the minimum concentration of methanol, necessary to keep all reaction products in solution and still allowing the formation of active iodine, is 80 %. All further experiments were conducted in a 8:2 methanol-aqueous buffer solution.

b) Influence of temperature.

The influence of the reaction temperature on the formation of iodobuprenorphine and side products was examined. To solutions of 5 mg buprenorphine.HCl (10 μmol) and 3,2 mg NaI (20 μmol) in 10 ml of the methanolic buffer, kept at a temperature of respectively 0°C, 25°C and 40°C, a solution of chloramine-T (10 μmol/200 μl phosphate buffer) was added and the mixture incubated for one minute. The reaction was stopped with sodium metabisulphite (40 μmol/1 ml phosphate buffer), the mixture extracted with 50 ml of chloroform and further purified as described below. At higher temperatures, lower yields and more side products were observed. Therefore, a temperature of 0°C was selected for the remaining series of experiments.

c) Influence of the chloramine-T concentration and reaction time.

The extent of iodination of buprenorphine was followed, using different concentrations of chloramine-T and during several time intervals. The amounts of buprenorphine (10 μmol) and NaI (12 μmol) were kept constant. Using increasing amounts of chloramine-T (10, 20, 30, 60 and 100 μmol), the oxidation was performed during 0.5, 1, 2, 5, 10 and 30 minutes respectively. The reaction products were all examined with

the HPLC procedure described below. It was shown that buprenorphine is extremely susceptible to oxidation by chloramine-T. High concentrations of the oxidant (> 30 μmol) result in the formation of other oxidation products, even for a short time of contact (30 seconds) with buprenorphine. Higher yields were obtained when the oxidant was added in small portions during the reaction. The best results were obtained using three equal amounts (5 μmol) of chloramine-T, added at time intervals of 4, 8 and 10 min.

d) Study of a two-step iodination procedure.

In another experiment, active iodine was formed separately from sodium iodide and chloramine-T. In a subsequent step, this mixture was allowed to react with buprenorphine.HCl. No better results however were obtained than for the one-step procedure.

e) Preparation and purification of iodobuprenorphine.

A solution of buprenorphine.HCl (10 μmol/10 ml) in 80 % MeOH- 20 % phosphate buffer pH 7.0 (0.066 M) was mixed with 100 μl of a sodium [$^{127}$I]iodide solution (12 μmol/ 100μl phosphate buffer). The temperature was kept at 0°C. 100 μl of a cold and freshly prepared chloramine-T solution (5 μmol/100 μl phosphate buffer) was added and the mixture vortexed for 30 sec. and kept at 0°C; after 4 minutes and 8 minutes, another aliquot of 100 μl of chloramine-T was added. After 10 minutes, 1 ml of $Na_2S_2O_5$ (40 μmol/1 ml phosphate buffer) was added to neutralise the chloramine-T. The mixture was adjusted to pH 8.6 with 10 ml of borate buffer and extracted with 50 ml of chloroform. 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 a 100 µl aliquot injected into the chromatograph. The mobile phase consisted of $CH_2Cl_2$ (69 %) - MeOH-diethylamine (99:1)(1 %) and n-hexane (30 %), at a flow-rate of 5 ml/min. This system allows an excellent separation of iodobuprenorphine and buprenorphine. By collecting the fractions of iodobuprenorphine from repeated HPLC separations, it was possible to obtain an amount useful for the identification of the molecule. The total yield of iodobuprenorphine was approximately 35 %. In Fig. 2, a typical HPLC chromatogram of the crude product is shown.

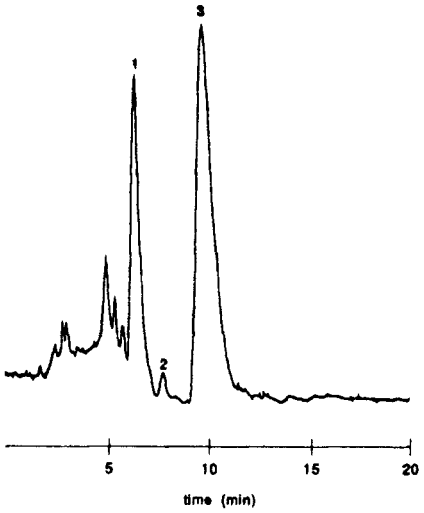


Fig. 2. HPLC-UV chromatogram, on a semi-preparative column, of the reaction products of the synthesis of [$^{127}$I]-iodobuprenorphine (1 = iodobuprenorphine; 2 = para-toluenesulfonamide; 3 = buprenorphine).

f) Identification of [$^{127}$I]iodobuprenorphine.

The reaction products were identified by mass spectrometry using the direct insert probe (DIP). A temperature program of

30°C/min from room temperature to 300°C was applied. The ionisation potential was set at 70 eV. The mass spectrum and the molecular structure of iodobuprenorphine is shown in Fig. 3.

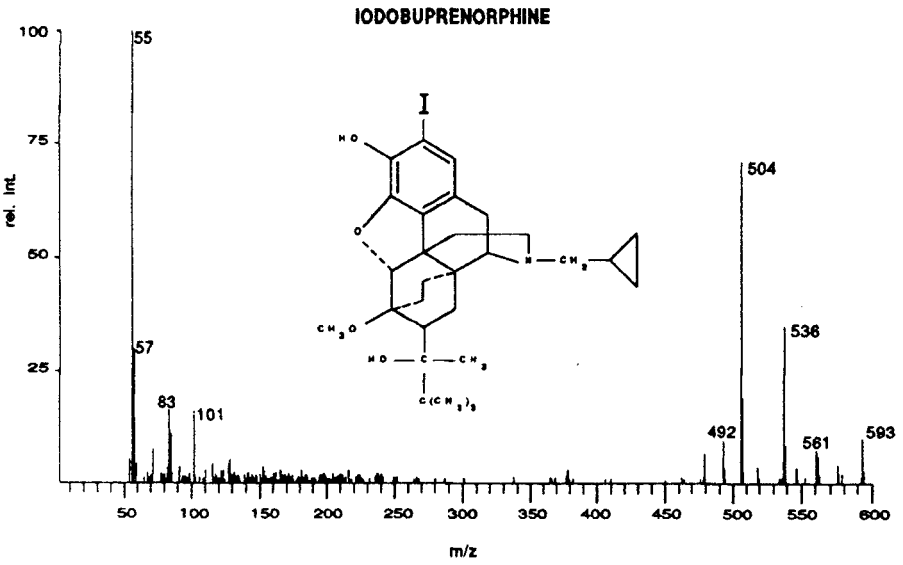


Fig. 3. Mass spectrum and molecular structure of [$^{127}$I]iodobuprenorphine.

The molecular ion is situated at m/z 593, indicating the incorporation of one iodine, most probably in the ortho position to the phenolic group. The mass spectrum is closely related to that of buprenorphine. Major fragment ions are m/z 561, 536, 504, 492, 101, 83, 57 and 55. The mass spectrum of the other two peaks of Fig. 2 allowed the identification of paratoluenesulfonamide (peak 2) and unchanged buprenorphine (peak 3).

g) Preparation and purification of iodobuprenorphine using Iodo-beads®.

The iodination of buprenorphine was also carried out with Iodo-beads®. The beads provide a gentle and more controllable method for iodination than by using free chloramine-T (11). Each uniform, non-porous polystyrene bead is

convalently modified with an oxidising agent (N-Chloro-benzenesulfonamide). The reaction conditions of the previous synthesis were scaled down in order to iodinate 2 µg (4 nmol) of buprenorphine.

To a solution of 200 µl phosphate buffer and 10 µl iodide (5 nmol), kept at room temperature, several beads (1 to 4) were added followed by 10 µl of the buprenorphine solution. The reaction was stopped at different time intervals (2, 10 and 20 min.) by simply removing the beads. The results of the synthesis were monitored with HPLC, using a Si-60 5 µm column (25 cm x 0,4 cm I.D.). The mobile phase consisted of $CH_2Cl_2$ (84 %) - MeOH-diethylamine (99:1)(1 %) and n-hexane (15 %), at a flow-rate of 1 ml/min.

It was shown that optimum iodination conditions were obtained by using only one bead and removing it already after 2 min. Although the iodination with Iodo-beads® gave controllable and reproducible results without any measurable side products, it was also shown that the polystyrene beads adsorb important amounts of non-reacted buprenorphine together with its iodo-derivative. Therefore this method was not selected for the subsequent radioactive synthesis.

B) Synthesis of [$^{125}$I]iodobuprenorphine

With slight adaptions to the procedure for the preparation of [$^{127}$I]iodobuprenorphine, the radioactive synthesis was done on a microscale. To a solution of 100 µl phosphate buffer, 10 µl (1 mCi, 0.5 nmol) of a sodium [$^{125}$I]iodide solution and 10 µl of a buprenorphine.HCl solution (30 v/v % MeOH; 1 mg/10 ml

= 2 nmol) were added. 10 µl (0.25 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 after 4 and 8 minutes. The chloramine-T was neutralised at time 10 min. by adding 10 µl of a aqueous sodium metabisulphite solution (1 mg/10 ml).

A solid phase extraction on a C18 column (3 ml) was selected for the isolation of iodobuprenorphine. The isolation of the labelled compound by extraction with chloroform was not suitable as it results in a high non-specific binding value in the immunoassay, due to the presence of co-extracted iodine. After conditioning the column with 3 ml of MeOH followed by 3 ml of borate buffer (pH 8.6), the reaction mixture was applied. The inorganic compounds were washed out with 6 ml of borate buffer. Elution was then performed with a MeOH-borate buffer mixture of increasing methanol concentration. The column was first washed with 15 ml of a mixture of methanolborate buffer (10:90) and then with 60 ml of a mixture of methanol-borate buffer (50:50) which elutes most of the side products. Finally 60 ml of a mixture of methanol-borate buffer (75:25) was used to isolate [$^{125}$I]iodobuprenorphine. This compound elutes between 10 and 30 ml but only the eluates between 12 and 18 ml were collected. This elution pattern was established in previous experiments by collecting 3 ml fractions on a fraction collector and measuring a 10 µl aliquot of each fraction for its radioactivity and its avidity to the antibodies. Fig. 4 demonstrates that the fractions with the highest radioactivity (fractions 34-36) also show the highest avidity (> 50 % binding) to the antibodies. These fractions were pooled and stored at 4°C.

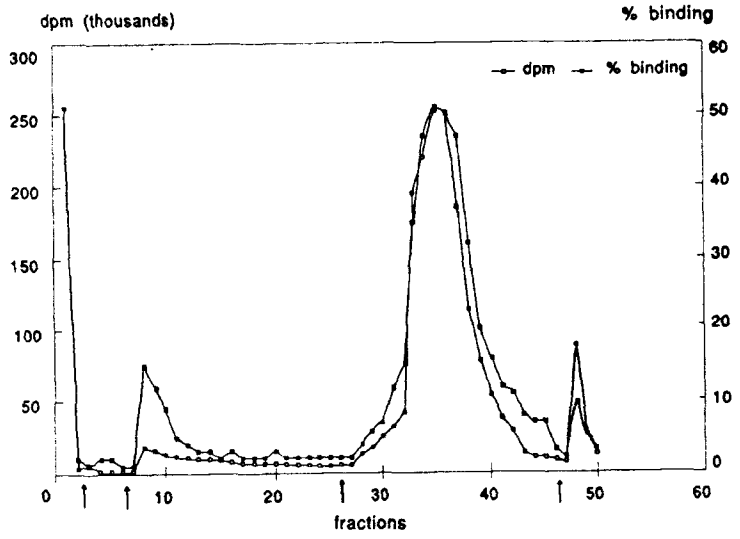


Fig. 4. Purification of the radioactive reaction mixture using solid phase extraction (RP-C18). The arrows indicate where the methanol concentration of the elution mixture increases (from 10 % up to 75 %).

C) Determination of the radiochemical purity and specific activity of the tracer

In order to improve the specific activity of the tracer and hence the sensitivity of the immunoassay, fractions 34-36, containing both buprenorphine and iodobuprenorphine, were further purified by HPLC. Due to the very low doses used in the radioactive synthesis and the low specific UV absorption of buprenorphine, it was necessary to use electrochemical detection for quantifying [$^{125}$I]iodobuprenorphine. The electrochemical detector was put into series with a gamma counter detector in order to check the radiochemical purity of the fractions eluted from the C18 column. The oxidation potential of the electrochemical detector was set at 750 mV and the sensitivity at 20 nA. The power supply of the gamma counter was

adjusted to 1.0 kV and the discriminator to 3.0 V. The eluate from the HPLC apparatus was led over the iodide crystal that was surrounded by a lead castle and further oxidised with the electrochemical detector. The combined fractions 34-36 were dried under a stream of nitrogen at 60°C and the residue dissolved in 500 µl mobile phase; 50 µl samples were injected into the chromatograph.

Chromatography was carried out on a LiChrospher CN, 5 µm column (25 cm x 0.4 cm I.D.) at a flow-rate of 1.0 ml/min. The optimum mobile phase, for separating buprenorphine and its iododerivative, consisted of phosphate buffer (pH 4.0, 0.02 M containing 10 mmol sodium 1-heptanesulphonate and 0.01 % of tetrabutylammonium-sulphate) (70 %) and acetonitrile (30 %). When monitored with the electrochemical detector, the chromatogram shows the presence of different impurities in the reaction mixture (Fig. 5). Iodobuprenorphine elutes at about 13

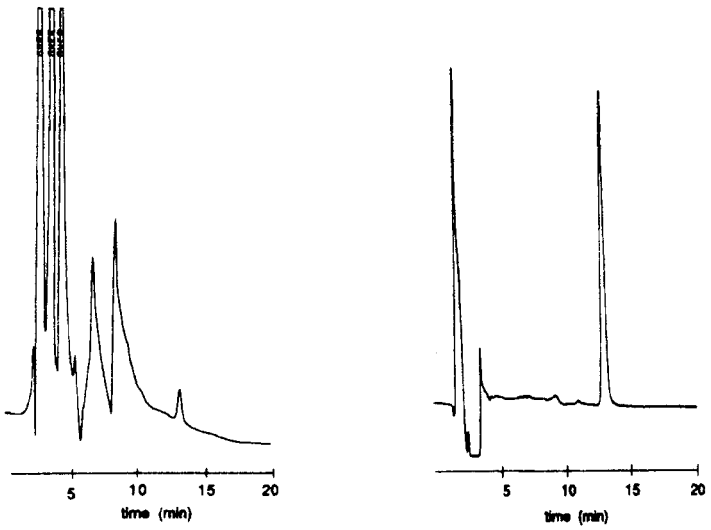


Fig. 5: chromatogram obtained with the electrochemical detector after injection of 50 µl of the combined fractions 34-36 (iodobuprenorphine, 4 ng/50 µl, elutes at 13 min.)

Fig. 6: chromatogram, with the electrochemical detector of a standard solution of [$^{127}$I]iodobuprenorphine (30ng/50 µl)

min. and is separated from other co-extracted impurities. Fig. 6 shows a chromatogram of a standard solution of [$^{127}$I]iodobuprenorphine. The chromatogram obtained with the gamma counter detector only shows one sharp peak (Fig. 7). No other radioactive compounds were detected.

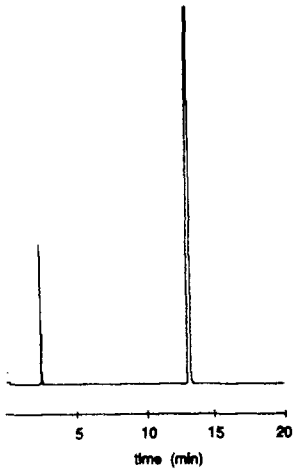


Fig. 7: chromatogram achieved with the gamma counter detector

The specific activity was determined by constructing a calibration graph of [$^{127}$I]iodobuprenorphine with the electrochemical detector. Therefore, aliquots of 1, 2, 10, 25 and 50 ng/50 µl mobile phase of [$^{127}$I]iodobuprenorphine were injected and the area under the curve measured. The concentration of the radioactive compound in the pooled fractions was calculated. The corresponding iodobuprenorphine peak was collected and the disintegration rate determined. The collected fraction counted 3,450,000 cpm/1 ng corresponding to 920 Ci/mmol.

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