6,7-DIMETHOXY-4-(4'-AMINO-3'[^{125}I]IODOBENZYL) ISOQUINOLINE : AN ISOQUINOLINE RECEPTOR PROBE.

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SUMMARY

Isoquinoline derivative, 6,7-dimethoxy-4-(4'-aminobenzyl) isoquinoline (DMABI) is synthetized. The iodinated derivative ^{125}I-DMABI of high specific activity (2175 Ci/mmol) labelled by Chloramine T method is separated from the unlabelled precursor by LH 20 chromatography and the probe is tested by its capacity to bind to rat intestinal membranes in vitro.

Our results show that the ^{125}I-DMABI is a useful tool for characterizing the isoquinoline intestinal binding site.

Key-words : Isoquinoline derivative, Isoquinoline binding site, Intestinal binding site, Iodine labelling, Iodine-125.

INTRODUCTION

The benzyl-4-isoquinolines exert spasmolytic activities in the rat duodenum (1), guinea-pig ileum (2,3) and rat aorta (4,5). As shown in pharmacokinetic study by using the

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0362-4803/86/070761-09$05.00

Received November 19, 1985
Revised February 10, 1986

^{14}C-labelled derivative, one of these compounds: 6,7-dimethoxy 4-(4'-chlorobenzyl) isoquinoline exhibits a high tissular affinity for liver, heart, aorta, cerebral vessels and intestine (6). Due to its low specific activity (20 mCi/mmol), the ^{14}C-PV 2 derivative is however not the best probe for studying the membranous isoquinoline binding site in vitro. The purpose of the present study is to obtain an isoquinoline probe of high specific activity; two approaches were found possible; tritiation and iodination. For tritiation, the labelled compound, obtained when the p-chlorobenzyl derivative is used in a halogen-tritium replacement reaction with tritium gas, requires extensive purification steps. We have thus chosen the second approach which consistsin the synthesis of a novel isoquinoline derivative 4'-aminobenzylated and its iodination by the Chloramine T method (9). Various groups (5,7,8) have shown that substitution in the 4'-benzyl ring does not alter its biological activity (3,4,8).

The present study describes the synthesis of 6,7-dimethoxy 4-(4'-aminobenzyl) isoquinoline (DMABI) and its precursors, iodination, purification, chromatographic behaviour and intestinal binding assay of this probe. The procedures used in the synthesis of DMABI and ^{125}I-DMABI are schematized in Figure 1.

EXPERIMENTAL

Materials and Methods

All chemicals are commercial origin and when possible reagent grade (Merck and Sigma). Carrier free Na^{125}I (IMS 300, 14.7 mCi/µg iodine, 2175 mCi/µatom iodine) is purchased from Amersham International plc (Buckinghamshire, England).

Purity control and reaction progress are checked by analytical TLC. Silicagel ready coated analytical TLC glass plates (Whatman LK6DF, 0.25 mm, 20 x 20 cm) are used. Spots are revealed by UV examination at 254 and 366 nm. Radioactivity on thin layer chromatographs is detected by autoradiography on Kodak Industrex AX4 films. Melting points are measured in capillary tube (Büchi-Totolli apparatus), the ^{1}H-nmr is recorded at 60 Mhz (Varian A 60 apparatus), internal reference, using $CDCl_3$ or (CD_3) CO TMS as solvents.

Purification of iodinated compound

Separation of the iodinated compound from this unlabelled precursor is conducted by gel filtration on Sephadex LH 20 (Pharmacia) (0.9 x 30 cm) eluted with ethanol : acetic acid (0.1%), 1 ml/min. Radioactivity is determined by counting in a Packard Autogamma 800 counter.

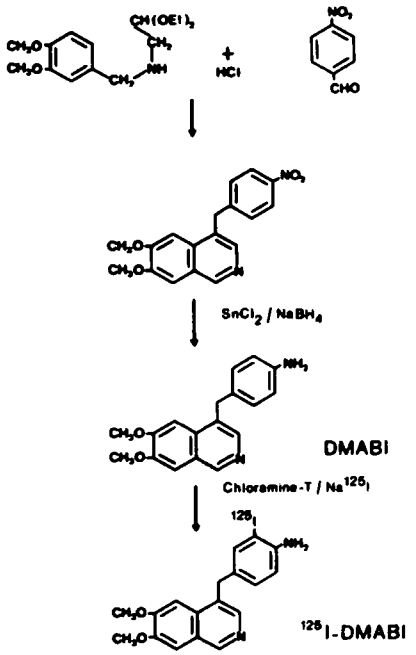

Figure 1 : **Chemical synthesis of DMABI and ^{125}I-DMABI**

6,7-Dimethoxy-4-(4'-nitrobenzyl) isoquinoline

6,7-Dimethoxy 4-(4'-nitrobenzyl) isoquinoline is prepared by condensation of 4-nitrobenzaldehyde (2.1 mmol) with the veratrylaminodiethylacetal (2.1 mmol) in the presence of concentrated hydrochloric acid (2.2 ml), according to Bouvier et al. (3). The oily residue is crystallized by treatment with acetone. The base is recristallized in 95% ethanol.

Yield: 46%; m.p.: 206°C; I.R. (KBr): 1570 (C=C), 1510 and 1340 cm^{-1} (NO_2); ^{1}H-nmr 3.88 (S, 3H, OCH_3), 4.08 (S,3H, OCH_3), 4.46 (S, 2H, CH_2), 6.96 (S, 1H, H_5), 7.32 (S, 1H, H_8), 7.42 (d, 2H, H at β of NO_2, J=9H_z), 8.22 (d, 2H, H at α of NO_2, J=9H_z), 8.27 (S, 1H, H_3), 8.93 (S, 1H, H_1).

Methanesulfonate salt: m.p.: 216-218°C (ethanol 95%); Anal. calculated for $C_{19}H_{20}N_2O_7S$ 418: C, 54.20; H, 4.79; N, 6.65. Found: C, 53.82; H: 4.92; N, 6.81.

6,7-Dimethoxy-4-(4'-aminobenzyl) isoquinoline (DMABI)

6,7-Dimethoxy-4-(4'-nitrobenzyl) isoquinoline (3.7 mmol) is dissolved by warming in 95% ethanol (160 ml). Powered stannous chloride (18 mmol) is added with stirring at room temperature. A suspension of sodium borohydride (2 mmol) in 95% ethanol (60 ml) is

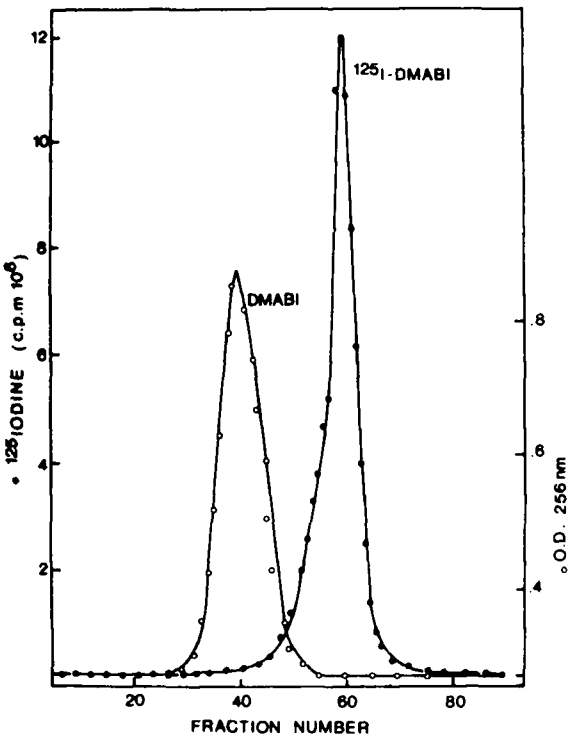

Figure 2 : **Separation of DMABI and ^{125}I-DMABI by Sephadex LH 20 chromatography**
A tracer quantity of ^{125}I-DMABI is added to 100 µg of DMABI in ethanol and applied to a column 0.9 x 30 cm of Sephadex LH 20 previously equilibrated with ethanol: acetic acid 0.1%. The column is eluted with the same solvent in 0.7 ml fractions. DMABI is measured by absorption at 256 nm.
A_{256} o—o ; ^{125}I •—•

introduced from a dropping funnel over a period of 30 minutes. The reaction mixture is heated under reflux for 30 minutes. After cooling, the ethanol is evaporated under reduced pressure. Ware (100 ml) is added followed by basification with sodium hydroxyde (3.5 M). The product is extracted with chloroform, the organic layer is washed with water to neutrality, dried over anhydrous sodium sulfate and the solvents evaporated under reduced pressure. The base is recristallized in 95% ethanol.

Yield: 88%; m.p.: 166-167°C; I.R. (KBr): 3460 and 3360 (NH_2); 1580 cm^{-1} (C=C);
^{1}H-nmr: 3.9 (S, 3H, OCH_3); 4.01 (S, 3H, OCH_3); 4.25 (S, 2H, CH_2); 6.68 (d, 2H, H at α of NH_2, J=9H_z); 7.01 (S, 1H, H_5); 1.28 (d, 2H, H at β of NH_2, J=9H_z); 7.29 (S, 1H, H_8); 8.35 (S, 1H, H_3);

8.98 (S, 1H, H_1)

Anal. calculated for $C_{18}H_{18}N_2O_2$ 294: C, 73.53; H, 6.17; N, 9.53. Found: C, 73.08; H, 6.49; N, 9.19.

6,7-Dimethoxy-4-(4'-amino-3'-[^{125}I]iodobenzyl) isoquinoline (^{125}I-DMABI)

To DMABI (6 µg in 6 µl of DMSO, 20 nmol) at room temperature is added sodium acetate buffer (24 µl, 1.0 M, pH 5.6), carrier-free Na^{125}I (2 mCi in 0.1 N NaOH) followed by chloramine T (6 µ l in 6 µl of H_2O, 21 nmol). After 1 minute the reaction is halted with sodium metabisulfite (8 µl in 8 µ l of H_2O, 42 nmol) and the mixture made basic by the addition of 1 N NaOH (5 µ l). The aqueous mixture is extracted into ethyl acetate (5 x 0.5 ml). The pooled organic extracts are concentrated under a stream of nitrogen.

Binding study.

Rat intestinal crude membranes are prepared in Tris-HCl buffer pH 7.5 (Tris-HCl 50 mM, $MgCl_2$ 5 mM, NaCl, 100 mM) by homogenization and differential centrifugation (10,11). The final pellet is resuspended to a concentration of 100 mg original wet tissue weight/ml buffer. The binding is conducted in Tris-HCl buffer pH 7.5 by addition of 100 µ l of crude membranes (30 to 60 ug of protein per assay) to a mixture of 50 µ l of ^{125}I-DMABI (0.15 u Ci) and competiting agents (50 µl) when specified. Incubation (60 min. at 30°C) is terminated by adding 1 ml of ice-cold Tris-HCl buffer containing 0.2% of BSA. After sample filtration (Whatman GF/C filters) and washing (2 x 12 ml ice-cold BSA-Tris buffer) the radioactivity bound to the membranes is measured in Packard Autogamma counter. Proteins are measured by Bradford method (12).

RESULTS AND DISCUSSION

The synthesis of unlabelled compound (DMABI) is afforted in good yield in two synthetic steps (46 and 88% respectively). The radioactive ligand (^{125}I-DMABI) is then readily prepared using chloramine T and carrier-free Na^{125}I. Extraction by ethyl acetate at pH 12 (Table 1), and separation of the unlabelled compound DMABI (26-55 fractions, Figure 2) from ^{125}I-DMABI (55-64 fractions) by LH 20 chromatography eluted with ethanol:acetic acid 0.1% make it possible to use a pure radioiodinated ligand. This is confirmed by the results of TLC analysis in several solvents systems (Table 2) which show a single radioactive product visualized by autoradiography, separated from the unlabelled compound. Due to the use of

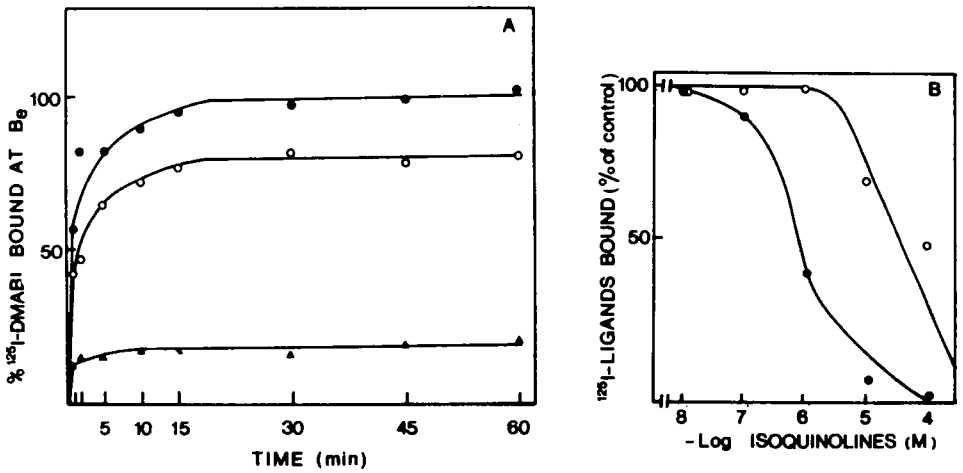

Figure 3 : A – Kinetic analysis of ^{125}I-DMABI binding to rat intestinal membranes

● Total binding ; ▲ Non-specific binding ; ○ Specific binding.

Specific binding is defined as that displaced by 10^{-4} M DMABI.

B – Displacement experiments with ^{125}I-DMABI by DMABI (●) and DMHBI (○).

Binding of ^{125}I-DMABI (0.75 nM) in standard assay conditions, proceeded for 60 min. in the presence of 10^{-8} to 10^{-4} DMABI or DMHBI

DMABI is 6,7-dimethoxy-4-(4'-aminobenzyl) isoquinoline

DMHBI is 6,7-dimethoxy-4-(4'-hydroxybenzyl) isoquinoline

carrier-free Na^{125}I and the complete purification of ^{125}I-DMABI a theoretical specific activity of 2,175 Ci/mmol is possible.

The iodinated probe is tested for their binding specificity to rat intestinal membranes. The following observation strongly suggest that the ^{125}I-DMABI is a good tool for the study of functional properties and pharmacological characterization of the intestinal isoquinoline receptor :

i/ Specific binding is time-dependent. The specific binding represent 80% of the total binding. Steady-state is reached at 15 minutes at 30°C and is maintained until 60 minutes. At equilibrium 122 fmol of ^{125}I-DMABI are specifically bound per milligram of proteins (Figure 3A).

ii/ The binding of ^{125}I-DMABI is displaced by a range of 10^{-8} to 10^{-4} M of unlabelled DMABI. The half of the displacement is obtained to 5.10^{-7} M (Figure 3B). Scatchard

Table 1 : **Percent of iodinated isoquinoline derivative extracted by different solvents**

Solvents	^{125}I-DMABI (% extacted)
Chloroform pH 7.5	33.1
Ethyl acetate pH 7.5	51.3
Ethyl acetate pH 12	92.6
Cyclohexane pH 7.5	76.2
Cyclohexane pH 12	89.3

analysis of the data gives a straight line suggesting that ^{125}I-DMABI interacts with a single population of non interacting binding site (concentration of binding sites: 102 ± 11 pmol/mg of protein) (11). The displacement by DMABI is better than observed with an other isoquinoline derivative that may be iodinated for example 6,7-dimethoxy-4-(4'-hydroxybenzyl) isoquinoline (DMHBI) (Figure 3B).

CONCLUSIONS

Labelled isoquinoline derivatives have been used in pharmacokinetics studies in vivo for characterizing the kinetics parameters and the metabolites of drugs : [6'-^{3}H] papaverine (13) [1-^{14}C] drotaverine (14) ^{14}C-PV 2 (6). The use of these low specific activity derivatives (5 to 1,600 mCi/mmol) just permits the observation of tissular affinity. Another low specific activity tritium labelled derivative: [3'-4'-methoxy-^{3}H] papaverine (209 mCi/mmol) has been used in vitro by Kimura et al. (15) for showing the binding of papaverine on protein located in the plasma rich membrane of the hog biliary duct. However the functional and pharmacological properties and the molecular identification of this protein binding are not described.

For studying this isoquinoline binding site we have developed an isoquinoline probe: the 6,7-dimethoxy-4-(4'-amino-3'-[^{125}I] iodobenzyl) isoquinoline directly derived from the 6,7-dimethoxy-4-(4'-chlorobenzyl) isoquinoline or PV 2 (2). The preliminary results related in this paper strongly suggest that the ^{125}I-DMABI is a good tool for this study. Moreover, this probe has been used for molecular characterization of the intestinal isoquinoline binding site by covalent photoaffinity labelling (11).

Table 2 : **Rf values obtained on Silica gel ready coated analytical glass plates (Whatman LK 6 DF), 0.25 mm, 20 x 20 cm)**

Solvents systems	DMABI	^{125}I-DMABI
$CHCl_3/CH_3OH/CH_3COOH$ 75/20/5	0.94	0.90
$CHCl_3/CH_3OH$ 93/7	0.88	0.85
$CHCl_3$/EtOAc/EtOH 40/40/20	0.73	0.65
$CHCl_3$/Cyclohexane/EtOH 50/30/30	0.74	0.63
$C_6H_6/CHCl_3/CH_3COOC_2H_5/CH_3COOH$ 20/20/20/2	0.30	0.04
$ButOH/CH_3COOH/H_2O$ 60/20/20	0.69	0.59

ACKNOWLEDGEMENT

Chemical part of this work is supported by a grant (CRE N° 85.1.024 - C. Viel) from Institut National de la Santé et de la Recherche Médicale (I.N.S.E.R.M.).

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