Table 3. Effect of mefloquine and primaquine on the metabolism of hexobarbital and zoxazolamine

Drug	Hexobarbital sleeping time (min)	Zoxazolamine paralysis time (min)
Control	33.4 ± 2.8 (9)	117.7 ± 9.2 (16)
Mefloquine HCl	36.6 ± 3.2 (8)	116.0 ± 9.3 (8)
Primaquine $diPO_4$	70.4 ± 4.5 (7)*	245.1 ± 9.7* (8)

The animals were pretreated for 40 min with either mefloquine HCl (50 mg/kg, p.o.) or primaquine $diPO_4$ (50 mg/kg, i.p.). Hexobarbital (120 mg/kg, i.p.) or zoxazolamine (60 mg/kg, i.p.) was then given. Control animals received an equal volume of normal saline. The result is expressed as mean ± SE from the number of rats indicated in parenthesis.

* $P < 0.05$ (from Control).

In conclusion, the present data suggest that the two quinoline derivatives (MQ and PQ) have different specificities in their inhibitory action on hepatic drug-metabolizing enzymes and that PQ is a more universal inhibitor of these enzymes than MQ.

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Characterization of [^{3}H]Ro 16-6491 binding to digitonin solubilized monoamine oxidase-B and purification of the enzyme from human platelets by affinity chromatography

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It is now generally accepted that the two forms of the FAD-containing enzyme monoamine oxidase (MAO*; EC 1.4.3.4.), namely MAO-A and MAO-B, are two structurally different proteins coded by distinct genes. A decisive breakthrough in clarifying the structure of MAO-A and MAO-B has come from molecular biology studies. Bach *et al.* [1] in fact have obtained two distinct cDNA encoding human liver MAO-A and MAO-B, showing that the two isoenzymes differ in primary structure, with a relatively high degree of homology (about 70%). Most of the evidence available suggests that the two isoenzymes have native molecular masses of about 120 kDa, being composed by two very similar or identical subunits. In contrast to previous findings [2, 3], recent studies indicate that each of these two subunits have covalently linked FAD as coenzyme [4].

MAO-A and MAO-B are both located in the outer mitochondrial membrane. Detergents are required for their

* Abbreviations used: MAO, monoamine oxidase; PEA, phenylethylamine.

extractions from membranes and the solubilized enzyme has a high tendency to be present in an aggregated form [5]. In addition, the agents generally employed for the solubilization of MAO, e.g. Triton X-100 and β-octylglucoside, have a marked effect on the enzyme catalytic properties [6, 7] and show other disadvantages, such as an inhibitory effect on the enzyme itself and produce a poor stability of MAO in the soluble preparation obtained [8, 9].

In the present paper we describe the use of digitonin as a suitable detergent for the effective solubilization of MAO-B from human platelets and frontal cortex membrane preparations and the characteristics of the solubilized enzyme using the selective MAO-B inhibitor [^{3}H]Ro 16-6491 [*N*-(2-aminoethyl)-*p*-chlorobenzamide HCl] as binding probe [10]. In addition, we describe an affinity chromatography method for human platelet MAO-B, using as affinity ligand a derivative of Ro 19-6327 [*N*-(2-aminoethyl)-5-chloro-2-pyridine carboxamide HCl], a compound in clinical trial belonging to a novel class of reversible and selective MAO-B inhibitors [11].

Materials and methods

Preparation and solubilization of human platelet and brain membrane preparations. Membranes from outdated human platelets and a crude mitochondrial fraction from human frontal cortices (autopsy brain, about 12 hr post-mortem) were prepared as reported earlier [10, 12]. The two preparations were stored at −70° (protein concentration 5 mg/ml) in Tris buffer (50 mM Tris, 130 mM NaCl, 5 mM KCl, 0.5 mM EGTA, 1 mM $MgCl_2$, pH 7.4). For solubilization of MAO-B, 1 volume of a digitonin (Wako, Osaka, Japan) solution (2%, w/v, in Tris buffer) was added to 1 volume of platelet membranes or brain mitochondria preparation (detergent/protein ratio, 4:1). The mixture was kept at 0° and gently homogenized four times every 15 min in a glass–Teflon homogenizer. Non-solubilized material was sedimented by centrifugation at 48,000 *g* (20 min at 4°). The supernatant was collected and stored at 4° or −20° until used. In some experiments three volumes of 2% digitonin were added to 1 volume of the solubilized preparation prior to use.

Binding of [^{3}H]*Ro* 16-6491. The binding of [^{3}H]Ro 16-6491 (sp. act. 17.8–16.8 Ci/mmol) to intact membrane preparation was performed as in Ref. 10. In digitonin-solubilized membranes [^{3}H]Ro 16-6491 binding was carried out by incubating (3 hr at 20°) aliquots of the preparations in the presence of various concentrations of the radioligand, in a final volume of 300 μl. In competition experiments, different concentrations of various drugs were added to the incubation mixture. At the end of the incubation, free radioactivity was adsorbed on to activated charcoal (50 μl/tube of a 10% solution in H_2O, containing 2% albumin). The tubes were then centrifuged in an Eppendorf Microfuge (12,000 *g* for 4 min) and 250 μl of the supernatant, containing the protein-bound ligand, were finally transferred to plastic vials for radioactivity counting. Non-specific binding was determined in parallel samples containing 100 μM selegiline. Binding kinetic data were calculated using a computer program [13].

Gel filtration chromatography. Samples (0.6 ml) of digitonin-solubilized membranes, previously incubated with 50 nM [^{3}H]Ro 16-6491, were applied to a Sepharose 6B glass column (Pharmacia, Uppsala, Sweden) (30 × 1 cm) equilibrated with Tris buffer containing 0.1% (w/v) digitonin. Elution was performed at 4° with the same buffer at a flow rate of 10 ml/hr. The UV absorbance of the eluate was measured at 224 nm. Five min fractions were collected in plastic vials and counted for radioactivity.

Synthesis of the affinity gel. The affinity gel for the purification of MAO-B was prepared as follows: 40 g of wet AH-Sepharose 4B (ω-aminohexyl agarose, Pharmacia) were reacted with 674 mg of Ro 19-5484 [*N*-(2-aminoethyl)-3-(2-iodo-acetamido)-2-pyridine carboxamide HCl] dissolved in 80 ml of coupling buffer (0.1 M $NaHCO_3$, pH 11) for about 20 hr at room temperature. The affinity gel was then extensively rinsed and finally stored at 4° in H_2O containing 0.02% (w/v) NaN_3.

Purification of human platelet MAO-B. For affinity chromatography, 20 ml of the affinity gel were poured in a glass column (i.d. 1.6 cm) and equilibrated with 0.2% digitonin in Tris buffer. A column containing 10 ml of uncoupled AH-Sepharose 4B was routinely used as pre-column in order to reduce the amount of contaminant proteins that bound to the affinity gel. The solubilized platelet preparation (digitonin/protein ratio, 4:1; 200 ml) was applied at 4° to the column system at 30 ml/hr. The precolumn was then excluded and the affinity gel was rinsed with 10 column volumes of 0.2% digitonin. MAO-B was eluted at 20° with a NaCl linear gradient (0.13–0.63 M) in Tris buffer containing 0.2% digitonin, at a flow rate of 30 ml/hr. The fractions containing the highest MAO-B activity were dialysed at 4° against 20 mM K-phosphate buffer, pH 7.4 containing 0.5 mM EGTA and 0.2% digitonin and then applied to a DEAE Sepharose CL-6B column (Pharmacia, 20 × 1.6 cm). The column was eluted with a linear gradient of K-phosphate buffer (20–250 nM, pH 7.4, containing 0.5 mM EGTA and 0.2% digitonin) at a flow rate of 30 ml/hr.

The different purification fractions were analysed by SDS–PAGE according to the method of Laemmli [14].

Radioenzymatic determination of MAO-B activity. MAO-B activity was determined radioenzymatically using 20 μM [^{14}C]phenylethylamine ([^{14}C])PEA, sp. act. 50.3 mCi/mmol, New England Nuclear, Boston, MA) as substrate [15].

Protein determination. Proteins were measured with the Pierce BCA Protein Assay (Pierce, Oud-Beijerland, The Netherlands). Values were corrected for the presence of digitonin.

Results

Solubilization of MAO-B with digitonin. Digitonin, even at the high concentrations used for the solubilization of MAO-B (see below), had only a negligible inhibitory effect on enzyme activity. In contrast, Triton X-100, β-octylglucoside, and the zwitterionic detergent CHAPSO [3-(3-cholamido-propyl)-dimethylammonio-2-hydroxy-1-propane sulfate] markedly inhibited MAO-B activity (data not shown).

After solubilization of platelet membranes and frontal cortex mitochondria with digitonin at a low detergent/protein ratio (4:1), about 90% of the [^{3}H]Ro 16-6491 binding activity was recovered in the soluble fraction. MAO-B enzymatic activity in the solubilized preparation was stable for several weeks upon storage at 0–4°.

Analysis of the 4:1 detergent/protein preparation, labelled with [^{3}H]Ro 16-6491, by gel-filtration chromatography on a Sepharose 6B column, showed that the radioactivity eluted in a non-homogenous broad peak (Fig. 1). However, when 3 volumes of 2% digitonin were added to the 4:1 preparation, the [^{3}H]Ro 16-6491-MAO-B complex eluted in a symmetrical peak with an apparent molecular mass of 70–80 kDa (Fig. 1). The UV absorption pattern also showed a shift towards lower molecular mass values (not shown). Higher amounts of digitonin had no further effect on the radioactivity elution pattern.

Binding of [^{3}H]*Ro* 16-6491 *to solubilized MAO-B.* In Fig. 2, the saturation curves of [^{3}H]Ro 16-6491 binding to platelet and frontal cortex-solubilized preparations are shown. The [^{3}H]Ro 16-6491 binding experiments described here were performed with the completely solubilized preparation obtained with high amounts of digitonin (see above). [^{3}H]Ro 16-6491 binding was of high affinity and the K_D values were similar to those observed in intact membranes ([10], Table 1). Non-specific binding was very low over a wide range of ligand concentrations (about

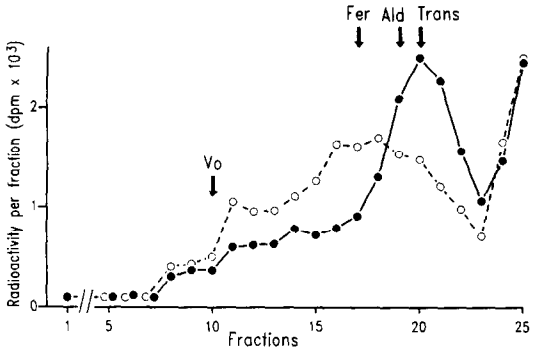

Fig. 1. Gel-filtration chromatography of [^{3}H]Ro 16-6491–MAO-B complex from platelet membranes solubilized with different amounts of digitonin. The solubilized preparations were incubated with 50 nM [^{3}H]Ro 16-6491 for 3 hr at 20° and subsequently applied to a Sepharose 6B column (30 × 1 cm). (○) Membranes solubilized with a detergent/protein ratio of 4:1 (1 vol. 2% digitonin + 1 vol. membranes at 5 mg protein/ml). (●) Membranes were solubilized as above and 3 vol. 2% digitonin were then added to the soluble preparation. Positions of void volume (V_o) and of marker protein elution are indicated by arrows. Fer (ferritin, 440 kDa); Ald (aldolase, 160 kDa); Trans (transferrin, 80 kDa). Data from a typical experiment.

20% of total binding at 200 nM). Binding equilibrium was reached after about 3 hr incubation at 20°, and [^{3}H]Ro 16-6491 dissociated from the solubilized membranes with a $T_{1/2}$ of about 2 hr (data not shown).

The binding of [^{3}H]Ro 16-6491 to digitonin-solubilized platelets was effectively prevented by inhibitors (Ro 16-6491, selegiline, almoxatone) or substrates (PEA and 1-methyl-4-phenyl-1,2,3,6-tetradropyridine) selective for MAO-B. Little or no effect on the binding was observed with MAO-A inhibitors (clorgyline, harmaline, amiflamine) or by the MAO-A preferential substrate 5-hydroxytryptamine.

Affinity chromatography of platelet MAO-B. The proposed structure of the Ro 19-5984-affinity gel is shown in Fig. 3. The concentration of ligand bound to the gel matrix after the coupling reaction, estimated by direct spectrometry of the gel [16], was about 1 μmol/ml of gel. No leakage of ligand from the affinity gel was observed.

MAO-B was normally purified starting from platelet membranes solubilized with the low detergent/protein ratio (4:1). When the preparation was applied to the affinity column, the adsorption of the enzyme to the gel was virtually quantitative and no apparent loss of MAO-B was observed during the column rinsing. It was found that unsubstituted AH-Sepharose 4B non-specifically retained relatively high amounts of proteins. The use of a precolumn containing the uncoupled gel during affinity chromatography greatly reduced the contamination of the affinity gel by other proteins. Interestingly, only minor amounts of MAO-B activity were retained by the AH-Sepharose 4B column.

The elution of MAO-B from the affinity column was obtained by means of a linear NaCl gradient. MAO-B eluted as a symmetrical peak and in this eluate a 40–50-fold enrichment in [^{3}H]Ro 16-6491 binding activity was obtained. Attempts to biospecifically elute MAO-B from the affinity column using an excess of PEA or of the inhibitors Ro 16-6491 and Ro 19-6327 were without success. After the affinity chromatography step, MAO-B could be further purified about 6-fold by ion exchange chromatography on DEAE Sepharose CL-6B.

Taking into consideration the B_{max} values of [^{3}H]Ro 16-6491 measured in the different purification fractions, it can be inferred that MAO-B could be purified <500-fold with a 35% recovery in respect to the starting material (Table 1).

The kinetic parameters of [^{14}C]PEA deamination in the different preparations obtained after the various purification steps are shown in Table 1.

SDS–PAGE analysis of the fraction obtained after affinity chromatography, showed an enrichment in a polypeptide band of 58 kDa. After the ion exchange step, most of the impurities still present in the affinity chromatography eluate, appeared to be removed and the purified MAO-B preparation was about 70–80% homogeneous.

Discussion

The present experiments indicate that digitonin is an excellent tool for the solubilization of MAO-B in the catalytically active form. Thus, after digitonin treatment of

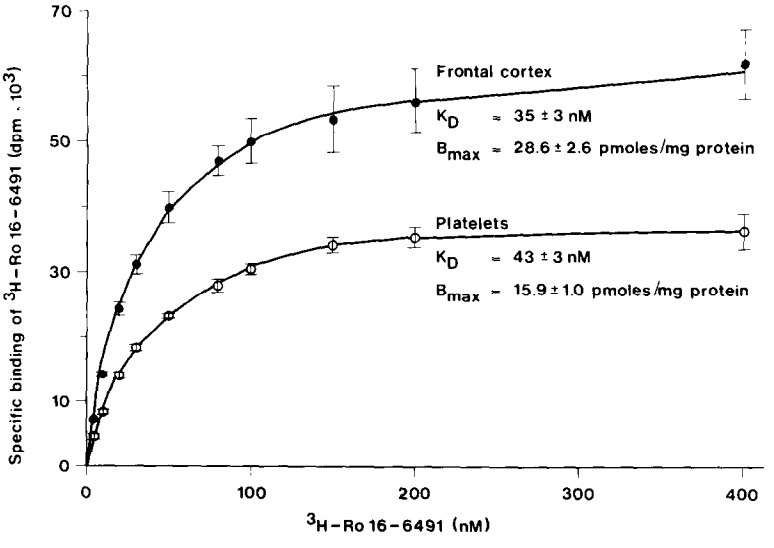

Fig. 2. Binding of [^{3}H]Ro 16-6491 to platelet membranes (○) and frontal cortex mitochondria (●), solubilized with digitonin. Values are means ± SE of three experiments performed in duplicate.

Table 1. Summary of the purification steps for monoamine oxidase-B (MAO-B) from human platelets

	[^{3}H]Ro 16-6491 binding		[^{14}C]PEA deamination		
	B_{max} (pmol/mg protein)	K_D (nM)	V_{max} (nmol/mg protein/min)	K_m (μM)	MAO-B recovery (%)
Membrane preparation	5.93	33.9	1.47	3.98	—
Digitonin preparation	11.84	18.8	1.97	2.86	87
Affinity chromatography	470.4	28.8	54.51	7.91	48
DEAE-Sepharose chromatography	3150.0	44.0	384.11	15.22	35

Results from a typical purification experiment.

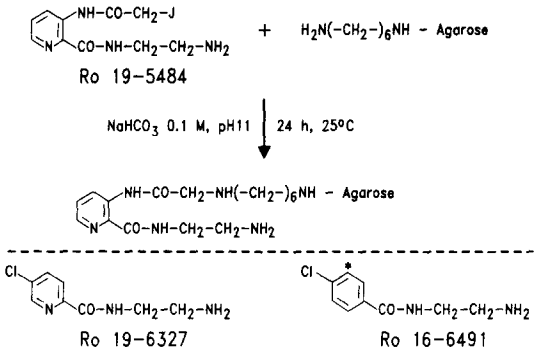

Fig. 3. Synthesis and proposed structure of AH-Sepharose 4B-Ro 19-5484 affinity gel. The structural formulas of the reversible MAO-B inhibitors Ro 19-6327 and Ro 16-6491 are also shown. *Position of tritium labelling in [^{3}H]Ro 16-6491.

human platelet membranes and frontal cortex mitochondria, the enzyme was recovered almost quantitatively in the soluble fraction. An advantage of the use of digitonin is that, in contrast to other detergents usually employed for the solubilization of MAO-B, the enzyme was not inhibited even in the presence of the high concentration of digitonin needed for extensive protein solubilization. In addition, we have observed that digitonin-solubilized MAO-B preparations appeared to be more stable upon storage than the preparations obtained with other detergents, e.g. Triton X-100, β-octylglucoside and CHAPSO.

Another interesting feature of digitonin is its ability to solubilize MAO-B in an apparently non-aggregated form of low molecular mass. It should be stressed that, at least for the tissues tested, the ratio of detergent to protein had to be raised to a critical value and very high amounts of digitonin were required for the complete solubilization of MAO-B.

We have previously reported the use of [^{3}H]Ro 16-6491 as reversible and selective radioligand for MAO-B in platelet and brain membranes [10]. [^{3}H]Ro 16-6491 appears to be a useful ligand in binding studies for the characterization and the measurements of MAO-B also in digitonin solubilized preparations. The binding of [^{3}H]Ro 16-6491 to solubilized MAO-B was specific and of high affinity even in the presence of high concentrations of digitonin and the characteristics of the binding were practically identical whether the MAO-B-solubilized preparation was obtained with a low detergent/protein ratio, or whether the enzyme was present in non-aggregated form.

Human platelets, which possess MAO activity only of the B type [17], have been widely used as source of this isoenzyme in man [18]. On the other hand, the amount of MAO-B present in these cells is very low. In fact, assuming a functional molecular mass of 120 kDa, it can be calculated from the B_{max} value for [^{3}H]Ro 16-6491 found in platelet membrane preparation (Table 1), that MAO-B accounts for less than 0.1% of the platelet membrane proteins.

Since affinity chromatography is a powerful technique for the purification of minor proteins, we have prepared an affinity gel for the purification of MAO-B by coupling a derivative of the reversible MAO-B inhibitor Ro 19-6327 to AH-Sepharose 4B.

The affinity chromatography of digitonin-solubilized MAO-B was quite satisfactory, producing a 40–50-fold purification of the enzyme in a single step. The adsorption of the enzyme depended on the presence of the coupled ligand, as the non-substituted AH-Sepharose 4B retained only minor amounts of MAO-B. This would indicate that the interaction between the affinity-gel and MAO-B was specific.

The purification of the enzyme is probably hampered by the fact that MAO-B, for unknown reasons, could not be eluted in a specific way from the affinity column by means of an excess of substrate (PEA) or of reversible inhibitors (Ro 16-6491 or Ro 19-6327). Due to the hydrophobicity of the gel spacer arm [19], several contaminating proteins were retained by the affinity column, being thereafter eluted with the salt gradient. The presence of the precolumn was therefore very important for lowering the amount of protein impurities that bound to the affinity gel.

Some observations can be made when comparing the enrichment in MAO-B obtained after the different steps, either by measuring the binding of [^{3}H]Ro 16-6491 or by measuring catalytic activity by the [^{14}C]PEA radioenzymatic method (Table 1). For example, B_{max} for [^{3}H]Ro 16-6491 increases twice after the digitonin solubilization while V_{max} for [^{14}C]PEA increases only by 1.4. Similarly, the final purification factor is <500-fold with the first method and about 260 with the second. Since it is conceivable that the conformation of the enzyme and some of its properties might be affected by the purification procedure, in our opinion this discrepancy is probably due to a decrease in the molecular turnover number of MAO-B after the solubilization step and in the purified preparation. Thus, this number appears to decrease from 249 to 166 pmoles of substrate/mg protein/min after the solubilization step, and to 122 in the purified preparation. Consistent with this, Houslay and Tipton [6] have shown that the solubilization of rat liver MAO with Triton X-100 altered the enzyme reaction pathway for benzylamine.

In conclusion, we think that the results shown in this paper represent a significant improvement for the study of MAO-B: (a) digitonin represents a good alternative as a detergent for the extraction and the study of MAO-B in solubilized preparations in which the enzyme is fully active

and present in a non-aggregated form; (b) [^{3}H]Ro 16-6491 is a reliable ligand probe for the investigation of the properties and for accurate measurements of MAO-B activity of different tissue preparations; finally (c) the affinity chromatography method described allows MAO-B to be purified by means of a relatively simple and rapid procedure, with an excellent yield in catalytically active protein.

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