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RESEARCH ARTICLES / BİLİMSEL ARAŞTIRMALAR

# Substrate Specificities of Monoamine Oxidase Isoforms

Gülberk UÇAR*°

***Substrate Specificities of Monoamine Oxidase Isoforms***

***Summary :*** *Monoamine oxidase (MAO), a mitochondrial enzyme which catalyzes the oxidative deamination of major neurotransmitter amines, exists in two forms, designated as MAO-A and B with different substrate specificities and sensitivities to the selective inhibitors. In spite of considerable progress in understanding the interactions of these forms with their preferred substrates, no general rules are yet available for the mechanism of their action towards the specific substrates. This study was planned as a preliminary investigation in order to detect the basic kinetic mechanisms of MAO isoforms towards their substrates. MAO was purified from rat liver mitochondrial pellets and human platelets. Substrate specificities of the isoforms were investigated by using p-tyramine, 5-hydroxytryptamine (serotonin) and benzylamine as substrates .The inhibition kinetics of the isoforms were tested by using the selective inhibitors of MAO-A and B forms such as clorgyline and pargyline.*

*p-Tyramine appeared as a mixed-type substrate since clorgyline or pargyline inhibited both the A and B forms when p-tyramine was used as substrate, suggesting that it would be a preferred non-selective substrate for distinguishing the MAO subtypes.*

*Benzylamine was found to be a selective substrate particularly for MAO-B isoform purified from human platelets. Pargyline caused a significant inhibition of the MAO-B activity of human platelets when benzylamine was used as substrate.*

*Serotonin was deaminated mainly by MAO-A and clorgyline inhibited the activity of this form isolated from rat liver homogenates. The present study indicated that the substrate specificities and kinetic behaviors of MAO isoforms might be essential for the design of the new selective MAO inhibitors.*

***Key Words:*** *Monoamine oxidase isoforms, monoamine oxidase inhibitors, serotonin, benzylamine, p-tyramine*

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***Monoamine Oksidaz İzoformlarının Substrat Özgüllükleri***

***Özet :*** *Major nörotransmitter aminlerin oksidatif deaminasyonunu katalizleyen ve mitokondri kaynaklı bir enzim olan monoamin oksidaz (MAO), farklı substrat özgüllükleri ve seçici inhibitör duyarlılığa sahip iki izoform (MAO-A and B) şeklinde bulunur. Bu formların, tercih ettikleri substratları ile ilişkileri konusunda dikkat çekici gelişmeler olmakla birlikte, özgül substratlarına karşı etki mekanizmaları hakkında henüz genel kurallar konulamamıştır.*

*Bu çalışma, MAO izoformlarının substratları ile ilişkilerinin temel kinetik mekanizmalarını tespit etmek üzere bir öncü araştırma olarak planlandı. MAO, sıçan karaciğer mitokondri pelletlerinden ve insan plateletlerinden saflaştırıldı. İzoformların substrat özgüllükleri substrat olarak p-tiramin, 5-hidroksitriptamin (serotonin) ve benzilamin kullanılarak ; inhibisyon kinetikleri ise klorgilin ve pargilin gibi seçici MAO-A ve B inhibitörleri yardımıyla araştırıldı.*

*P-Tiramin substrat olarak kullanıldığında MAO-A ve B formlarının pargilin ya da klorgilin ile inhibe olmaları nedeniyle p-tiramin karışık-tip substrat olarak tanımlandı ve bu substrattan, spesifik inhibitörler kullanıldığında MAO isoformlarının ayırımda bir non-selektif substrat olarak yararlanılabileceği kanısına varıldı.*

*Benzilamin'in, özellikle insan plateletlerinden saflaştırılan MAO-B formu için seçici substrat olarak davrandığı ve benzilamin varlığında platelet kaynaklı MAO-B izoformunun pargilin ile inhibe olduğu bulundu.*

*Serotoninin , büyük oranda MAO-A ile deamine olduğu ve sıçan karaciğeri kaynaklı MAO-A izoformunun serotonin varlığında klorgilin ile inhibe olduğu görüldü.*

*Bu çalışma, MAO izozimlerinin substrat özgüllükleri ve kinetik davranışlarının yeni ve seçici MAO inhibitörlerin dizaynı açısından önem taşıdığını gösterdi.*

***Anahtar kelimeler :*** *Monoamin oksidaz izoformları, monoamin oksidaz inhibitörleri, serotonin, benzilamin, p-tiramin*

## 1. INTRODUCTION

Mammalian monoamine oxidase (MAO,EC 1.4.3.4) is an integral flavin-containing enzyme of the outer mitochondrial membranes of neuronal, glial, and other cells, which is responsible for regulation and metabolism of major monoamine neurotransmitters such as serotonin (5-OH tryptamine), adrenaline, nor-

* Hacettepe University, Faculty of Pharmacy, Department of Biochemistry, 06100, Ankara, TURKEY.
° Correspondence

adrenaline and dopamine[1]. It is also involved in the biodegradation of exogenic amines such as benzylamine, tyramine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenyl pyridinium ($MPP^+$) and a "Parkinsonian producing neurotoxin" [1]. It is found in two different forms designated as MAO-A and MAO-B ( with molecular weights of 59.700 and 58.800, respectively) which are encoded by two different genes[2] and distinguished by different substrate specificities and sensivities to the selective inhibitors[3]. MAO-A was suggested to be inhibited by e.g. clorgyline and prefers serotonin and nor-epinephrine as substrates, whereas MAO-B is inhibited by e.g. pargyline and prefers dopamine and benzylamine as substrates[4]. Although both forms of the enzyme are present in the liver and brain of mammals, it was suggested that only the B-form is present in human platelets and only the A-form is present in placenta[5].

It is now well established that oxidation of substrates by monoamine oxidases is coupled to the reduction of flavin adenine dinucleotide (FAD) cofactor and gives an imine as the product which is hydrolysed spontaneously to yield the corresponding aldehyde and ammonia[5]. The reaction catalyzed by monoamine oxidase enzymes for most substrates can be summarized as follows:

$$RCH_2NR_1R_2 + H_2O \rightarrow RCHO + NHR_1R_2 + H_2O_2$$

Over the past ten years, efforts have been directed to the design, synthesis and study of new monoamine oxidase inhibitors (MAOIs) for the treatment of various mental and neurological disorders[6]. MAO inhibitors of the new generation are usually characterized by their relative specificities for the MAO subtypes and in some cases by the reversibility of their actions. Despite considerable progress in understanding the interactions of the two enzyme forms with their preferred substrates and inhibitors, no general rules are yet available for the rational design of potent and selective inhibitors of MAO. Preferential MAO-A inhibitors have been recognized as therapeutically useful antidepressants while MAO-B inhibitors have been found to be benefical in the treatment of Parkinson's disease and Alzheimer[6-11].

Since understanding of the interactions of the two MAO isoforms with their preferred substrates and inhibitors will help to supply some basic information about the substrate binding and inhibitory properties of the isoforms, in the present study the substrate specificities of MAO isoforms purified from rat liver mitochondria and human platelets were investigated using p-tyramine, benzylamine and serotonin as substrates for MAO. Inhibition kinetics of both isoforms which interacted with their specific or non-specific substrates were also studied using their selective inhibitors such as clorgyline and pargyline. The results were compared with those reported earlier.

## 2. MATERIALS and METHODS

### 2.1.Materials

Clorgyline, pargyline, vanillic acid, 4-aminoantipyrine, peroxidase (type II, horseradish), p-tyramine hydrochloride, benzylamine hydrochloride, serotonin creatinine sulfate, TRIS, EDTA, CHAPS, and Triton X-100 were purchased from Sigma-Aldrich, Co. All other reagents were of analytical grade.

### 2.2.Purification of MAO from the liver homogenates

MAO was purified from the rat liver ( 100-150 g) (Ethics Committee #: 2001/25-4) according to the method of Holt[12] with some modifications. Homogenates of liver were prepared with 1:25 (w/v) in ice-cold potassium phosphate buffer , pH 7.4 with a polytron mechanical homogenizer. Homogenates were centrifuged at 1000g, at 4°C for 15 min. and the supernatant was used as the source of MAO and kept at -70°C. Since MAO-A activity decreased rapidly fol-

lowing homogenization, freshly prepared homogenates were used in kinetic studies of MAO-A activity. These crude homogenates were used as the MAO source for preliminary kinetic assays.

Mitochondrial MAO was purified by isolation of mitochondria from liver homogenates. Liver tissue ( 5-8 g) was homogenized 1:40(w/v) in 0.3 M sucrose. Following centrifugation at 1000 g for 10 min., the supernatant was centrifuged at 10 000 g for 30 min. to obtain crude mitochondrial pellet. The pellet was incubated with either CHAPS of 1% or Triton X-100 of 1% at 37°C for 60 min. and centrifuged at 1000 g for 15 min. The pellet was resuspended in 0.3 M sucrose and layered onto 1.2 M sucrose, centrifuged at 53 000 g for 2 hours and resuspended in potassium phosphate buffer. It was kept at -70°C until used.

### 2.3. Purification of MAO-B from human platelets

Venous blood samples were pooled in stoppered laboratory vacutainer tubes containing sodium citrate as anticoagulant. Platelet-rich plasma (PRP) was prepared by sequential centrifugation: blood (300-500 mL) was centrifuged at 200 g for 15 min. at room temperature; supernatant was aspirated, recentrifuged at 1200 g for 10 min. and kept. The pellet was resuspended in 0.5 mL of TRIS buffer (15 mM TRIS-HCl, 140 mM NaCl, 10 mM EDTA), pH 7.4 and centrifuged at 10 000 g for 10 min. Supernatants were pooled. Platelet counts were determined on aliquots of pooled PRP diluted in Isoton II and counted twice on a thrombocounter ( Coulter Electronics, STKS)[13]. The kinetic behavior of MAO-B activity of the pooled PRP was determined as described below by using benzylamine as substrate ( in the concentration range of 0-10 mM) and the results were expressed as nmol/ $10^8$ platelets/h.

### 2.4 Measurement of MAO activity

Total MAO activity was measured spectrophotometrically according to the method of Holt[12]. The chromogenic solution which is prepared for inclusion in the assay mixture consisted of 1 mM vanillic acid, 500 µM 4-aminoantipyrine, 4 $U/mL^{-1}$ peroxidase in 0.2 M potassium phosphate buffer, pH 7.6. Chromogenic solution was prepared daily and kept at 4°C until used.

A standard assay mixture contained 167 µL chromogenic solution, 667 µL substrate (500 µM p-tyramine , 450 µM benzylamine or 500 µM serotonin) and 133 µL potassium phosphate buffer, pH 7.6. The mixture was preincubated at 37°C for 10 minutes before the addition of enzyme. Reaction was initiated by addition of the homogenate (100 µL) and absorbance increase was followed at 498 nm at 37°C for 60 minutes. Molar absorption coefficient of 4654 $M^{-1}cm^{-1}$ was used to calculate the initial velocity of the reaction.

### 2.5 Selective measurement of MAO-A and MAO-B activities

Rat Liver homogenates were incubated with the mixed substrate p-tyramine at 500 µM to measure MAO-A and at 2.5 mM to measure MAO-B; 450 µM for benzylamine and 500 µM for serotonin following inhibition of MAO with inhibitors ( 50 µM) or at 500 µM to measure total MAO when no inhibitor had been included. Substrate concentrations were chosen with respect to our pervious studies on MAO activity of bovine and sheep liver homogenates (unpublished data).

Aqueous solutions of clorgyline or pargyline ( 50 µM) were added to homogenates at the ratio of 1:100 (v/v) so the final inhibitor concentrations were 500 nM. Homogenates were incubated with inhibitors at 37°C for 60 minutes prior to activity measurement.

In order to test the dependence of reaction upon enzyme concentration, a range of volumes of inhibitor-treated homogenates (25-100 µL) was made up to total volumes of 100 µL with potassium phosphate buffer and then incubated with p-tyramine, at 500 µM or 2.5

mM, to assay MAO-A and B activities, respectively.

### 2.6. Protein determination

Protein contents were determined according to the method of Bradford[13] with bovine serum albumin used as standard.

## RESULTS and DISCUSSION

MAO was purified from the mitochondrial extracts of rat liver homogenates and from human platelets. For the extraction of the membrane-bound enzyme from the mitochondrial pellets, Triton X-100 and CHAPS were tested at the various concentrations, temperatures and incubation times. Since Triton X-100 caused a significant reduction in enzyme activity, CHAPS was used as the detergent for extraction procedure. Optimum detergent concentration, incubation time and temperature were found to be 1%, 60 minutes and 37°C, respectively.

The specific MAO activity was found to be 43 nmol/mg protein for rat liver homogenates when p-tyramine was used as substrate. MAO activity of human platelets were calculated as 24 nmol/$10^8$ platelets/h when benzylamine was used as substrate. The specific MAO activity of the liver homogenates which were not treated with CHAPS was found to be decreased to 28 nmol/ mg protein when p-tyramine was used as substrate. This result showed that the extraction of the membrane-bound enzyme with detergents from the mitochondrial pellets were essential for the complete isolation of MAO from liver tissues.

The initial rates of tyramine metabolism by both MAO-A and B were found to increase linearly with increasing enzyme concentration (Figure 1). Rat liver homogenates were preincubated at 37°C and pH 7.4 for 60 minutes with either clorgyline or pargyline before the assay so that the kinetic parameters of the other could be determined. Further preincubation had no additional inhibitory effect on the MAO activity. This incubation period seemed to be required for their reaction with the enzyme-inhibitor complex followed by an irreversible reaction to form a covalent adduct in agreement with the previous reports[15].

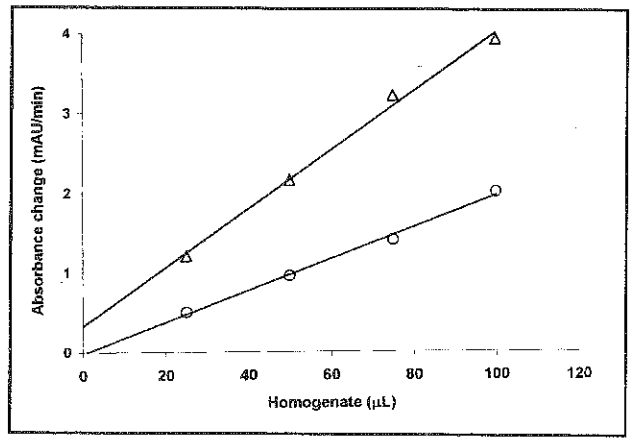


Figure 1. Dependence of reaction velocity upon the enzyme concentration. Liver homogenates were incubated with clorgyline (50 µM) (Δ) to inhibit MAO-A or pargyline (50 µM) (0) to inhibit MAO-B prior to incubation with p-tyramine at 500 µM to measure MAO-A and 2.5 mM to measure MAO-B, respectively.

Kinetic parameters of rat liver MAO-A and MAO-B towards tyramine is shown in Table 1 and Figure 2. $K_m$ and $V_{max}$ values of rat liver MAO-A and MAO-B were found to be 0.02 mM and 23.81 nmol/h/mg protein ; 0.06 mM and 22.22 nmol/h/mg protein, respectively when p-tyramine was used as substrate.

**Table 1.** Kinetic parameters of rat liver MAO-A and B activities towards p-tyramine*

| Inhibitor | Form remaining | $K_m$ (mM) | $V_{max}$ (nmol/h/mg protein) |
|---|---|---|---|
| None | A and B | 0.035±0.002 | 55.22±2.80 |
| Pargyline | A | 0.021±0.001 | 11.01±2.30 |
| Clorgyline | B | 0.045±0.002 | 40.00±3.20 |

* Values represent the mean ± SE of six measurements.

Rat liver homogenates were incubated for 60 minutes at 37°C with either water or 50 µM inhibitor before activities were determined. P-Tyramine concentrations were chosen as 10-100 µM for MAO-A and 50-500 µM for MAO-B.

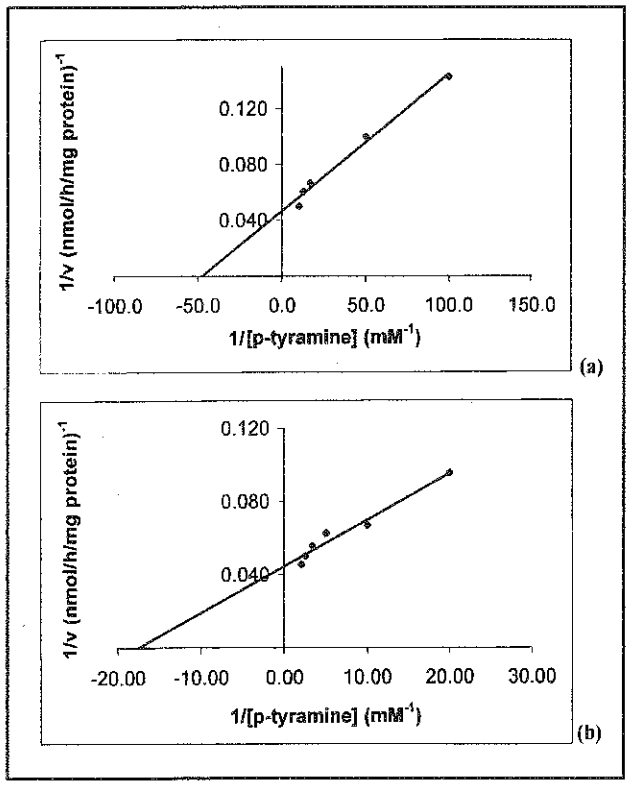


Figure 2. Lineweaver-Burk plots of the deamination of p-tyramine catalyzed by rat liver MAO-A (a) and B (b).
Rat liver homogenates were preincubated either with clorgyline or pargyline prior to incubation with tyramine (10-100 µM for MAO-A and 50-500 µM for MAO-B).

Reversibility of the inhibiton of rat liver MAO by the inhibitors was assessed by repeated washing, centrifugation and resuspension after preincubation of the enzyme and inhibitor for 60 min at 37°C. Under these conditions both clorgyline and pargyline were found to be irreversible inhibitors of MAO subtypes when p-tyramine was used as substrate since no activity was regained after 4 reversibility cycles.

Although p-tyramine was reported as a mixed-type substrate for MAO isoforms previously[5,15], the present study showed that it had a greater affinity to MAO-A than the MAO-B purified from rat liver (Table 1).

Figure 3 shows the effects of clorgyline and pargyline on the p-tyramine metabolism by MAO isoforms. Inhibitors reduced MAO-A and B activities by 97% and 94%, respectively at 50 µM concentrations, indicating that the combined use of selective inhibitors with a non-selective substrate such as p-tyramine is suitable for distinguishing MAO subtypes.

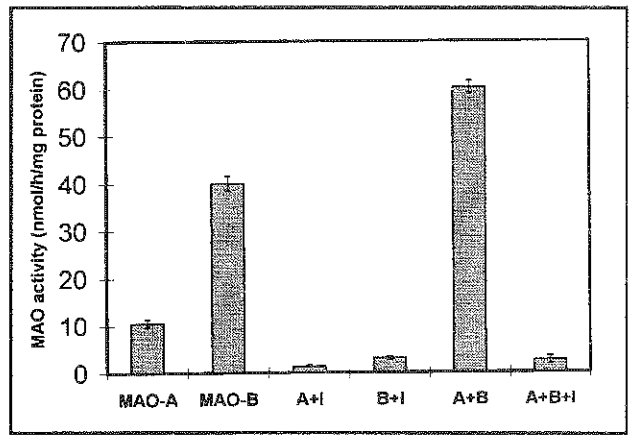


Figure 3. Effect of selective inhibitors on rat hepatic MAO activity. Deamination of p-tyramine ( 500 µM for MAO-A and 2.5 mM for MAO-B) was determined following preincubation of homogenates with pargyline (MAO-A); clorgyline (MAO-B); pargyline followed by clorgyline (A+I); clorgyline followed by pargyline (B+I); water (A+B); and water followed by clorgyline+pargyline (A+B+I).
Results are the mean±SE from six assays.

Figure 4 shows the benzylamine metabolism by rat liver MAO isoforms. $K_m$ and $V_{max}$ values were calculated as 1.42 mM and 1.77 nmol/h/mg protein for MAO-A and 0.58 mM and 0.06 nmol/h/mg protein for MAO-B indicating that benzylamine behaved as a selective substrate for rat liver MAO-B isoform. Since platelets have been previously reported to contain only the B form of MAO[18], benzylamine metabolism of human platelets by MAO isoforms was also determined. It was found that platelets had only MAO-B activity with the $K_m$ and $V_{max}$ values calculated as 0.19 mM and 48.18±4.26 nmol/ $10^8$ platelets/h (Fig 5). When platelets were subjected to 60 minutes of preincubation with pargyline prior to determination of the enzyme activity by using of benzylamine as substrate, a complete inhibition was observed (Figure 6). This data indicated that benzylamine appeared as a substrate for the platelet-derived MAO-B form.

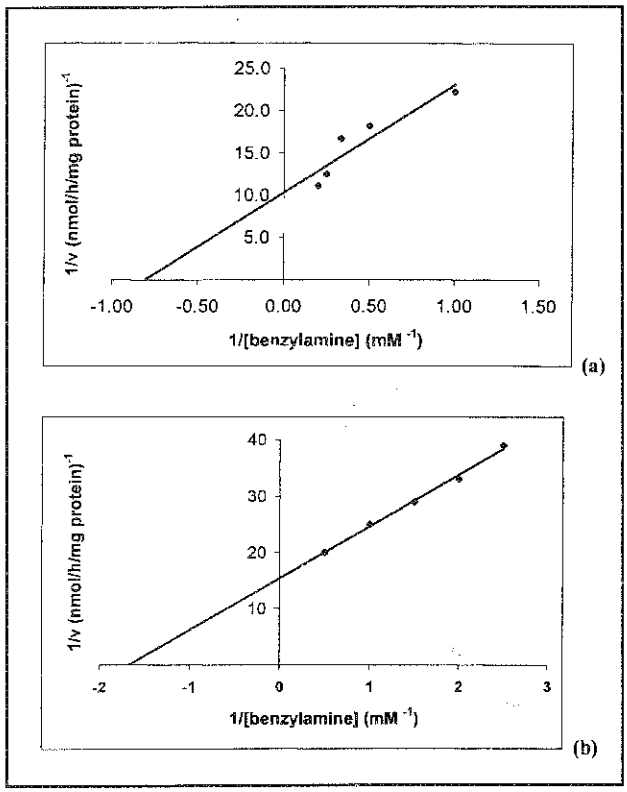


Figure 4. Lineweaver-Burk plots of the deamination of benzylamine catalyzed by rat liver MAO-A (a) and B (b).
Rat liver homogenates were preincubated either with clorgyline or pargyline prior to incubation with benzylamine (100-500 µM for MAO-A and 40-300 µM for MAO-B).

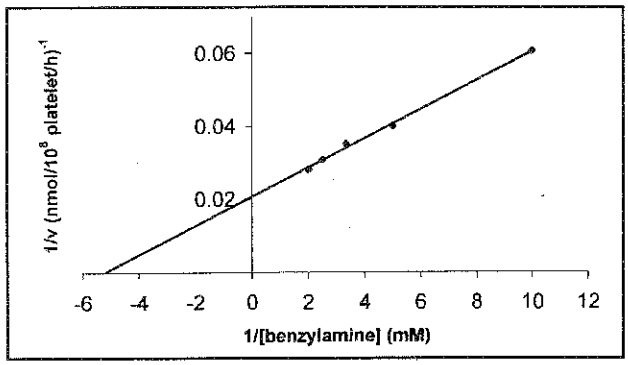


Figure 5. Lineweaver-Burk plot of the deamination of benzylamine (100-500 µM) catalyzed by platelet MAO-B isoform.

Serotonin was deaminated by MAO-A with $K_m$ and $V_{max}$ values as 0.44 mM and 50 nmol/h/mg protein, respectively (Figure 7) while it has no significant activity towards MAO-B. Since preincubation of rat liver homogenates with clorgyline ( 50 µM) prior to incubation with serotonin (500 µM) caused a significant inhibition and pargyline had no inhibitory effect on MAO activity of homogenates, it was concluded that serotonin was mainly metabolized by rat liver MAO-A ( Figure 8).

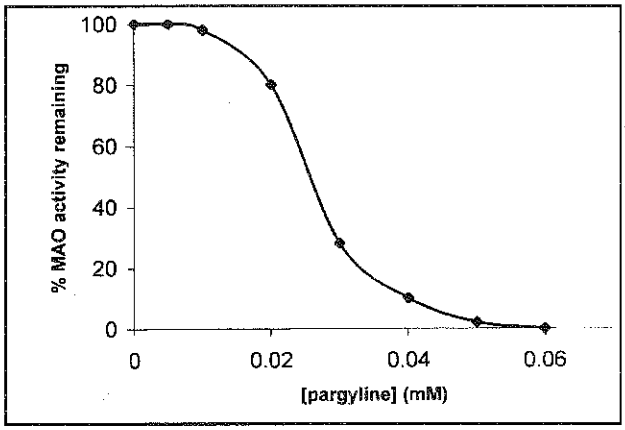


Figure 6. Inhibition of the deamination of benzylamine by pargyline. Liver homogenates were preincubated with various concentrations of pargyline at 37°C for 60 minutes prior to incubation with benzylamine (450 µM).

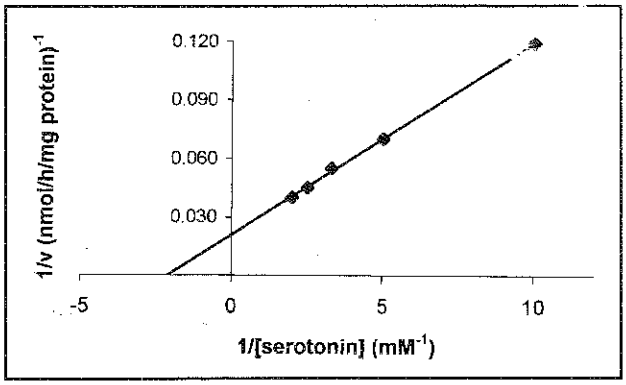


Figure 7. Lineweaver-Burk plot of the deamination of serotonin catalyzed by rat liver MAO-A. Homogenates were preincubated with clorgyline ( 50 µM) prior to incubation with serotonin.

Earlier studies with liver and brain homogenates have shown clogyline to interact with both types of MAO to form an initial non-covalent complex, and selegiline had a lower $K_i$ value towards MAO-B when dopamine was used as substrate[3] whereas clorgyline was suggested to be a irreversible MAO-A inhibitor and (-)-deprenyl and pargyline acted as the irreversible inhibitors of MAO-B when p-tyramine and tryptamine were used as substrates[16-19]. 100 nM clor-

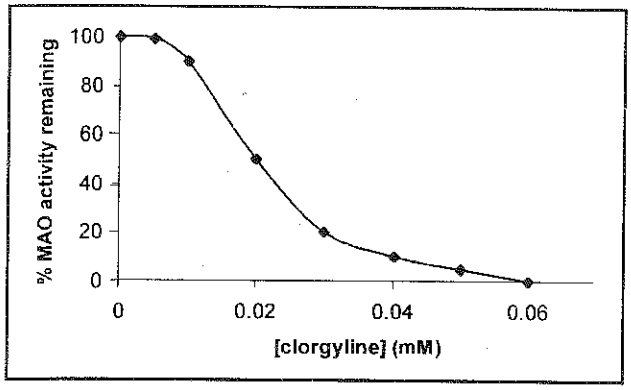


Figure 8. Inhibition of the deamination of serotonin by clorgyline. Liver homogenates were pre-incubated with various concentrations of clorgyline at 37°C for 60 minutes prior to incubation with serotonin ( 500 μM).

gyline has been reported to inhibited MAO-A of rat intestine while 1 μM clorgyline inhibited both A and B forms when 150 μM tyramine was used as substrate[16]. Fowler[3] indicated that ratio of A and B form activities were dependent upon the concentration of the substrate used. According to these reports, results of the present study were in good agreement with the findings suggesting that affinities of MAO isoforms to various specific or non-specific substrates were affected by a number of factors such as enzyme source, substrate concentration and the existence of inhibitors.

## CONCLUSION

Considering the different $K_m$ values found for MAO isoforms of liver homogenates and human platelets, MAO isoenzymes from both tissues seemed to have different affinities toward p-tyramine, benzylamine and serotonin as substrates. The present study also indicated that those differences in the kinetic parameters of MAO isoforms might be important for their specificities towards the selective substrates in accordance with the previous reports[20-22]. Significantly lowered $K_m$ values of rat liver MAO-A found towards serotonin suggested the role of MAO-A isoform in the deamination of serotonin in the brain. MAO-B might be the major isoform responsible for the metabolism of benzylamine in liver, platelets or other tissues. Although rat liver had both MAO-A and B activities towards p-tyramine, the data showed that $K_m$ value of MAO-A towards tyramine was lower than that of the B form.

As a result, differences in substrate specificities of MAO isoforms might have a physiological importance for the design of the novel selective MAO inhibitors.

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