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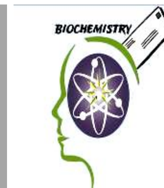

The Effect of the anticonvulsant (S)-2-[4-(3-Flouro-Benzyloxy) Benzylamino]-Propionamide on Monoamine Oxidase (A&B)

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ABSTRACT

The effect of the potent anticonvulsant the alanine derivative (S)-2-[4-(3-flourobenzyloxy)-benzylamino] propionamide (FCE 26743) on MAO-B from ox liver mitochondria and human platelets and MAO-A & –B from Human brain mitochondria has been studied. This compound involves replacement of glycinamide of the parent (2-n-pentylamino acetamide) compound with alanine and replacement of the pentyl moiety with a phenyl ring substituted in the *para*-position by a 3-flouro-benzyloxy group. (FCE 26743) was not metabolized to any extent by either form of the enzyme from any of the preparations used. For human brain mitochondrial MAO-A and –B In the absence of preincubation, FCE 26743 was found to be a competitive inhibitor with the IC_{50} values of approx 80 μM and 0.26 ± 0.024 μM respectively. This showed FCE 26743 to be > 300 times more potent as an inhibitor of MAO-B than of MAO-A. After preincubation with FCE 26743 for 30 min at $37^{O}C$, no significant time-dependent inhibition of human brain MAO-A was found. However the degree of inhibition of MAO-B increased significantly. This was confirmed by the extended time-courses studies which showed a rather rapid increase in the degree of inhibition of MAO-B. The IC_{50} values for the inhibition of 5-HT and PEA oxidation after preincubation were > 80 μM and 0.079 ± 0.009 μM, for human brain MAO-A and -B, respectively

FCE 26743 inhibited human platelet MAO-B before incubation with IC_{50} value of 0.16 ± 0.023 μM and after preincubation of FCE 26743 and the enzyme at $37^{O}C$ for 30 min the IC_{50} value was 0.064 ± 0.014 μM. The inhibition studies with ox liver mitochondrial MAO-B indicated that without enzyme-inhibitor preincubation, the inhibition of this form of the enzyme was also competitive and shows that FCE 26743 is > 500-times more potent inhibitor for human brain MAO-B than for ox liver MAO-B. Furthermore, MAO-B from ox liver showed little or no significant time-dependent inhibition by this compound.

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1. Introduction:

The involvment of neurotransmitter systems in the cellular mechanism of epilepsy has been suggested [1]. Because of the vital role that Monoamine oxidases play in the inactivation of neurotransmitters, MAO dysfunction is thought to be responsible for a number of psychiatric and neurological disorders For example, unusually high or low levels of MAOs in the body have been associated with schizophrenia [2,3] and depression [4]. Monoamine oxidase is a well known enzyme in pharmacology, since it is the substrate for the action of a number of monoamine oxidase inhibitor drugs. In fact, MAO-A inhibitors act as antidepressant and antianxiety agents, whereas MAO-B inhibitors are used alone or in combination to treat Alzheimer's and Parkinson's diseases [5], although they are often last-line treatment due to risk of the drug's interaction with diet or other drugs.

The potent anticonvulsant FCE 26743, the alanine derivative (S)-2-[4-(flourobenzyloxy)-benzylamino] propionamide belongs to a series [6] of analogues of milacemide (2-(n-pentylamino) acetamide) a glycine derivative with atypical anti-epileptic and potential psychotropic properties and differs from milacemide in that alaninamide residue replaces the glycinamide moiety, and in the presence of a phenyl ring substituted in the *para*-position by a 3-flouro-benzyloxy group. FCE 26743 showed potent protective properties in a number of models of epilepsy in rodents, such as bicuculline-induced convulsions and lethality, maximal electroshock and kainic acid-induced seizures and status epilepticus [7]. Introduction of a benzyloxy group in the *para* position of a phenyl ring in MAO substrates and inhibitors has been shown to transform the substrates into inhibitors [8]. The potential interest of combining both MAO-B inhibition and anti-epileptic activity in the same molecule remains to be established. Since the amino acid alanine, unlike glycine, does not possess an anticonvulsant activity, an investigation of the mechanism of action of this compound with MAO may help in understanding the anticonvulsant behavior of this group of compounds.

In vitro studies with rat brain homogenates,[9] showed FCE 26743 to be a potent inhibitor of MAO-B and to weakly inhibit MAO-A, whereas its enantiomeric counterpart FCE 28073 was approxamately 10-times less potent as a MAO-B inhibitor and 7-times more potent as a MAO-A inhibitor than FCE 26743. These workers also showed that the results of *in vitro* preincubation studies and of experiments involving the *in vitro* and "*ex vivo*" dilution of brain homogenates experiments were consistent with FCE 26743 acting as an irreversible MAO-B inhibitor. In contrast, the time-course of "*ex vivo*" inhibition of rat brain MAO-B with this compound was a typical of short acting inhibitor. The proposed mechanism that may account for the apparently contrasting results as in (Scheme-1)

(Scheme-1)

In the first step the enzyme (E) and the inhibitor (I) are in rapid equilibrium with the enzyme inhibitor complex (EI), as for a classical reversible inhibition. Then a second step occurs through initial single-electron transfer from the nitrogen nonbonding electrons to the oxidized FAD followed by α-proton abstraction [10]. This would lead to the formation of an intermediate imine. In the case of classical

substrates the imine is subsequently hydrolysed to yield the corresponding aldehyde. In the present case, one may assume that the imine reacts with a group, possibly -SH, present in the MAO protein with production of enzyme-inhibitor adduct (termed EI*), resulting in MAO inhibition. This adduct might slowly dissociate to regenerate the imine, which would be then hydrolysed. In an *in vitro* system, the equilibrium would be strongly displaced towards the formation of the enzyme-inhibitor adduct, EI*, so that the compound behaves as an irreversible inhibitor. In contrast, under *in vivo* conditions, the aldehyde formed by hydrolysis could be cleared, favoring the reversibility of the reaction from EI* back to the imine, with FCE 26743 behaving as a short-acting "*ex vivo*" inhibitor as a result. Additional experiments are needed to establish whether FCE 26743 acts as a slow-binding inhibitor, or is metabolized to some degree by MAO-B to form an adduct with the enzyme, which would dissociate under *in-vivo* conditions. In an attempt to understand more precisely the mechanism of action of this compound, in vitro studies of FCE 26743 as a MAO-(A and –B) substrate and inhibitor were carried out using preparations from human and ox.

2. Experimental procedures

2.1. *Material*:

Benzylamine HCL and 5-Hydroxytryptamine creatine sulphate (5-HT) were obtained from Sigma Co. 5-Hydroxytryptamine (5-HT) [side chain-2-^{14}C] creatine sulphate and phenylethylamine - (ethyl-1 ^{14}C) hydrochloride (PEA) were obtained from Amersham International or New England Nuclear. FCE 26743 was kindly given by Pharmacia-Farmitalia Carlo Erba. All other chemicals were standard laboratory chemicals and were of analytical reagent grade whenever possible.

2.2. *Methods:*

Human brain mitochondria and human platelets were prepared by the methods of Cesura *et al* [11, 12]. Human brain was obtained within 12 hours of death and transferred to the laboratory on ice. Ox liver mitochondria were prepared by the method of Salach [13]. The mitochondrial pellet obtained was suspended in a small volume of 0.1M potassium phosphate buffer, pH 7.2 and stored at -20°C until use for MAO-B or -A activity. The Aldehyde Dehydrogenase (ALDH) was partly purified from ox liver by a modification [14] of the method of Deitrich [15] and IU of activity is defined as the amount that catalyses the formation of 1µ mol product / min at 37°C in the presence of 500µM NAD^{+}and 3mM acetaldehyde. The molar extinction coefficient (ε) of NADH at 340 nm was taken to be 6.22x10^{3} M^{-1}.cm^{-1} [16].

All enzyme assays were performed at 37°C and pH 7.2.[17]. The activity of MAO-A was determined using the substrate 5-hydroxytryptamine, by the coupled spectrophotometric assay in which the formation of NADH is followed continuously at 340 nm as the aldehyde product is further oxidized by ALDH [14]. MAO-B activity was examined spectrophotometrically at 250nm by directly monitoring the formation of benzaldehyde from benzylamine [18]. The molar extinction coefficient (ε) of benzaldehyde at 250 nm was taken to be 13.8 x 10^{3} M^{-1}.cm^{-1} [19].

The behavior of FCE 26743 as a substrate of MAO was determined by measuring the change in absorbance at 283 nm using direct spectrophotometric assay, the molar extinction coefficients for the expected

aldehyde product were determined to be 4.358×10^3 ($l.mol^{-1}.cm^{-1}$) from our previous studies (pending).

The effects of FCE 26743 on the activity of MAO-A and -B were determined using the radiochemical assay by the method of Otsuka & Kobayashi [21] as modified by Fowler [20]. using 5-HT and PEA as the selective substrates for MAO-A and -B, respectively. IC_{50} values (concentration of the inhibitor giving 50% inhibition) were determined at zero-time and after 30 min preincubation of the enzyme and inhibitor using the computer program Kaleidagraph .

The kinetic behavior of FCE 26743 on MAO-A &-B from both species were determined spectrophotometrically, by coupled or direct assays by varying the substrate concentration (5-HT and benzylamine, respectively), in the presence of several fixed concentrations of the inhibitor. The K_i values were calculated by fitting the data of the apparent K_m/V_{max} values (slopes) versus the inhibitor concentrations using the computer program Mac-CurveFit. Extended time courses of inhibition of human brain mitochondrial MAO-A &-B and ox liver mitochondrial MAO-B were determined spectrophotometrically by monitoring the product formation by either coupled (at 340 nm, using 500 μM 5-HT as a substrate) or direct (at 250 nm with 250 μM benzylamine as substrate) assays in the presence of the indicated concentrations of FCE 26743. The kinetic parameters K_i and k_{+2} were determined by analysing the graphs of the time courses of inhibition of each form of the enzyme as described in Tipton *et.al.* [23]. according to the equations:

$$\ln (P_\infty - P_t) = \ln P_\infty - k't \qquad (1)$$

$$k' = \frac{k_{+2}}{1 + (K_i / i)[1 + (s / K_m)]}$$

Where P_t is the product concentration at any time t, and P_∞ is the final product concentration when the reaction has ceased, s represents the substrate concentration, i represents the inhibitor concentration and K_i is the dissociation constant for the non-covalent complex. Thus a graph of 1/k' against 1/i will give a straight line with a slope of $(1 + s / K_m) K_i / k_{+2}$ that intersect with the y axis at a point corresponding to $1/k_{+2}$. However, the computer program (Mac-Curve Fit) was used to determine k' and P_∞ values in the present work. The double reciprocal plots are used only for illustrative purpose

3. Results:

3.1. Interaction of MAO-A and -B with FCE 26743 as a substrate:

Human brain and ox liver, mitochondria and human platelets MAO-B were assayed spectrophotometrically at 283 nm with FCE 26743, as a substrate. But no change in the absorbance could be detected. Thus FCE 26743 was not metabolised at any significant rate by MAO in any of these preparations. (No data shown).

3.2. Inhibition Studies:

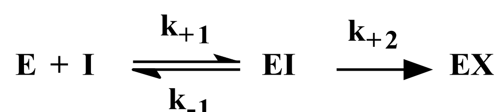
Studies on the effects of FCE 26743 on the activities of human brain mitochondrial MAO-A and -B and MAO-B from ox liver mitochondria and human platelets (as shown in Fig 1&2 and is taken as a representative of the other enzyme form used) show that In the absence of preincubation, FCE 26743 was found to inhibit, human brain mitochondrial MAO-A and -B with IC_{50} values of 80 μM and 0.26 ± 0.024 μM, respectively and human platelet MAO-B with IC_{50} value of 0.16 ± 0.023 μM. Thus FCE 26743 is a much better inhibitor for MAO-B than MAO-A

of the same species. No significant time-dependent inhibition of human brain MAO-A was found when preincubated with FCE 26743 for 30 min at 37°C, however the degree of inhibition of MAO-B increased significantly. The IC_{50} values for the inhibition of 5-HT and PEA oxidation after preincubation were > 80 µM and 0.079 ± 0.009 µM, for human brain MAO-A and -B, respectively (Table 2) and 0.064 ± 0.014 µM for human platelet MAO-B.

(Figs 3a and 3b) show the kinetics of inhibition of ox liver mitochondrial MAO-B by FCE 26743 and is taken as being representative of the other enzyme forms used. FCE 26743 was shown to be linearly competitive towards the amine substrate for all the enzyme forms used, ox liver mitochondrial MAO-B and human brain mitochondrial MAO-A and B and human platelet MAO-B. The K_i values for the inhibition of the different forms of the enzyme were determined and shown in (Table 1).

3.3. Time courses of inhibition and determination of kinetic parameters:

Fig 4a shows Extended time-courses of the inhibition of human brain mitochondrial MAO-A by FCE 26743 in the presence of 5HT, determined spectrophotometrically at 340nm in the presence of FCE 26743 and is taken as being representative of the other enzyme forms used. The graphs would be consistent with FCE 26743 behaving as a time-independent inhibitor that initially interacts competitively with respect to the amine substrate for MAO-A and B used in these studies, and it is a much weaker inhibitor of MAO-A from human brain mitochondria than its MAO-B form. The inhibition of ox MAO-B required FCE 26743 concentrations in the range 80 - 250 µM which is close to that used for human brain mitochondrial MAO-A (40 - 200 µM) and much higher than the ones used for human brain mitochondrial MAO-B (0.02 - 0.08 µM). MAO-B from human platelets behaved similar to that of the human brain enzyme. The low K_i and k_{+2} values obtained by Analysing the progress curves as described above and shown in Fig 4b, according to the simple mechanism:



$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{+2}} EX$$

show that such analysis is not applicable for these types of reactions.

4. Discussion:

The anticonvulsant compound FCE 26743 has been reported to be a potent and highly selective inhibitor of MAO-B from rat liver and brain [9]. It belongs to a series of analogues of milacemide (the oxazolidinone derivatives) and differs from milacemide in that an alaninamide residue replaces the glycinamide residue and in the presence of linked aromatic groups instead of the pentyl group. If this compound were to act as a substrate for MAO, it might be expected to yield alaninamide, rather than glycinamide which might argue against the slow delivery of glycine to the brain being an important factor in the anticonvulsant activities of the milacemide series of compounds. The compound FCE 26743 was tested as a substrate for ox liver and human brain mitochondria *in vitro.* The activity of the mitochondria was assessed by following the change with time of the UV absorption at 283 nm, no product formation could be detected. Moreover, when MAO-B from human platelets was tested in the same way, there was no change in the absorbance. Thus FCE 26743 was not metabolised at any significant rate by MAO in any of these

preparations. However, the possibility of alternative products being formed cannot be excluded. This result would rule out the possibility that accumulation of the aldehyde product is involved in the inhibition of MAO by FCE 26743, suggesting that the inhibition is due to the molecule itself. This is in agreement with the results obtained by Strolin Benedetti [9] from the *in vitro* studies in which the aldehyde was found to be roughly 10-times less potent as an inhibitor of rat brain MAO-B than FCE 26743 itself.

The results of the present inhibition studies indicate that FCE 26743 is far more potent inhibitor of the B-form of monoamine oxidase than the A-form. MAO inhibition has been used as a successful approach in the treatment of neuropsychiatric disorders [24]. The involvement of neurotransmitter systems in the cellular mechanism of epilepsy has been suggested and higher brain monoamine levels have been shown to reduce seizure susceptibility [25]. The MAO-B inhibitory property of FCE 26743 might contribute to its anti-epileptic activity by increasing brain dopamine levels during long-term treatment. Furthermore, the decreased formation of H_2O_2 in brain might result in a lower level of oxidative stress in epileptic patients. Since FCE 26743 does not affect the phenobarbitone-inducible cytochromes P450 [22], this compound might have the added advantage of not affecting the levels of the natural radical scavenger vitamin E. In the present *in vitro* inhibition studies with human brain mitochondria the selectivity of FCE 26743 towards MAO-B, as measured by the ratio IC_{50} MAO-A / IC_{50} MAO-B, was greater than 300 and 1000 after 0 and 30 min incubation, respectively. Whereas the degree of inhibition of MAO-A did not change after 30 min enzyme-inhibitor preincubation, the degree of inhibition of MAO-B increased significantly, which is consistent with FCE 26743 being a reversible inhibitor for MAO-A and a slow- binding reversible inhibitor of MAO-B. The inhibition ratios, IC_{50} MAO-A / IC_{50} MAO-B, for the enzymes from rat brain mitochondria were reported by Strolin Benedetti *et al.*[22] to be greater than 400 and 700 after 2 and 60 min incubation, respectively. Comparison of the reported ratio values for rat brain with the values for human brain obtained in the present studies, show that the ratio values with incubation were higher for human brain and the values before incubation were lower. Although the studies on the time-dependence of inhibition shown above indicated that the inhibition of MAO-A was unaffected by preincubation with FCE 26743, there was an increase in the strength of inhibition when MAO-B from human brain was incubated with this compound. This was confirmed by the extended time-courses studies which showed a rather rapid increase in the degree of inhibition of MAO-B, which appeared to be complete after about 15 min after which time there was no significant further time-dependent effect, despite incubation being continued for a total of 5h. This finding is in close agreement with the *in vitro* inhibition studies[9] with rat brain MAO-B where the percentage inhibition increased in the first 10 min but then did not change, or even decreased slightly, over the next 50 min. The behavior of MAO-B from human platelets was also similar to that of the human brain enzyme.

The results of the kinetic studies to determine the inhibition constant K_i of FCE 26743 towards ox liver mitochondrial MAO-B indicate that without enzyme-inhibitor preincubation, the inhibition of this form of the enzyme was also competitive and shows that FCE 26743 is

500-times more potent inhibitor for human brain MAO-B than for ox liver MAO-B. Furthermore, unlike MAO-B from the other species studied, that from ox liver showed little or no significant time-dependent inhibition by this compound. Such results emphasise the need for caution in extrapolating results obtained from one species to the possible behavior in another. It is also intriguing that the MAO-B in one of the human brain samples studied behaved atypically (see Table 2) in having a low sensitivity to FCE 26743, resembling more closely the enzyme from ox liver than that from the other human brain preparations. It will be necessary to screen other brain samples for inhibition by this compound before one can conclude that a genetic variation is involved.

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Table 1. K_i Values for the Inhibition of MAO from Human Brain and Ox Liver, Mitochondria by FCE 26743

Enzyme preparation	Substrate	K_i (µM)
- Human Brain mitochondria:		
MAO-A	5-HT (b)	58.3 ± 11.3
	5-HT (c)	34.0 ± 10
MAO-B	Benzylamine (a)	0.028 ± 0.02
	PEA (c)	0.019 ± 0.01
- Ox liver mitochondria:		
MAO-B	Benzylamine (a)	27.76 ± 3.1

The K_i values were calculated as described above using the computer program Mac-CurveFit. The assay methods used were: (a) direct spectrophotometric assay at 250 nm. (b) Coupled spectrophotometric assay at 340 nm. (c) Radiochemical assay. The values are the mean ± S.E.M. of three determinations.

Table 2. The IC_{50} Values for the Inhibition of MAO from Human, Brain Mitochondria and Platelets by FCE 26743

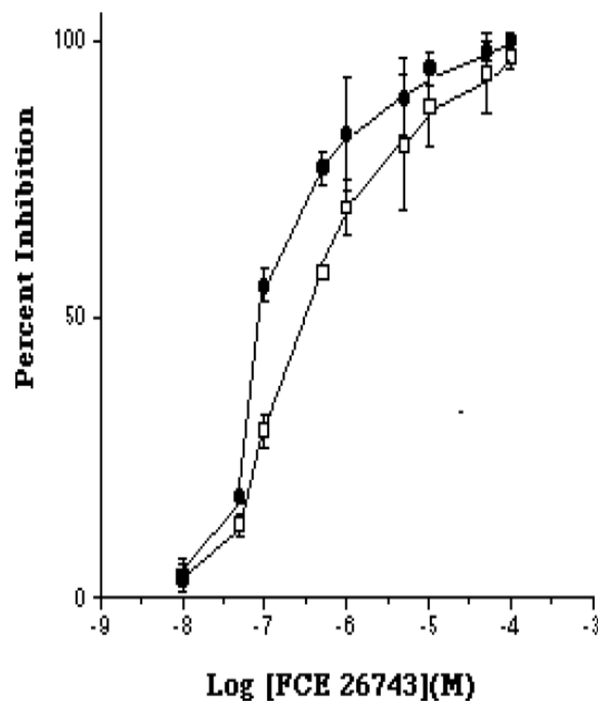
Enzyme preparation	IC_{50} (µM) Without preincubation	IC_{50} (µM) 30 min preincubation
- Human brain mitochondria:		
MAO-A	approx. 80	approx. 80
MAO-B	0.26 ± 0.024	0.079 ± 0.009
A-Typical Human brain:		
MAO-A	88 ± 8	88 ± 8
MAO-B	79 ± 15	79 ± 15
Human Platelets:		
MAO-B	0.16 ± 0.023	0.064 ± 0.014

MAO activities were determined radiochemically at 37°C with 20 µM 2-phenylethylamine or 100 µM 5-hydroxytryptamine, as substrates for MAO-B and-A, respectively.

Figure 1. The Effects of FCE 26743 Concentrations on the Activities of Human Brain Mitochondrial Monoamine oxide p9se

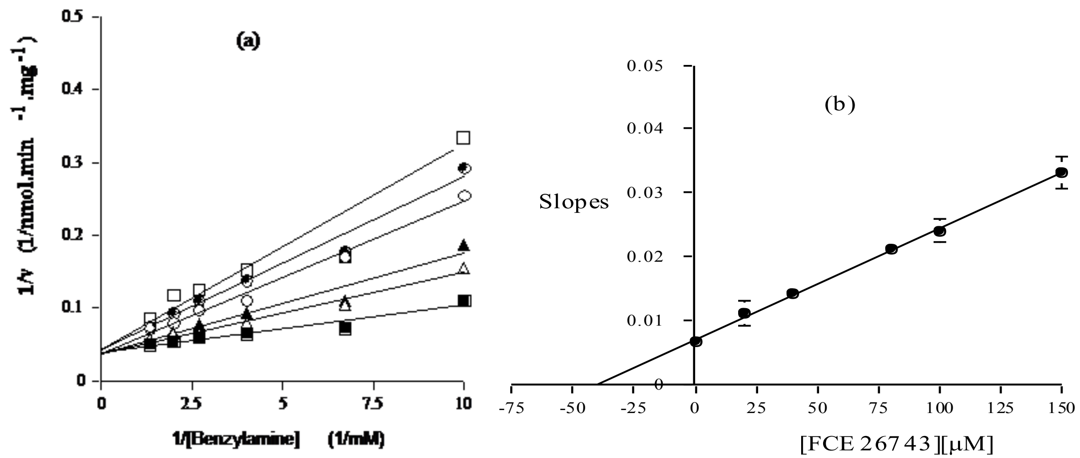
The enzyme preparation (0.2 mg/ml) was incubated with the indicated concentration of FCE 26743 for (□) 0 time or (●) 30 min before activity was determined radiochemically towards (a) 100μM 5-HT or (b) 20 μM PEA. Percentage inhibition was calculated with respect to samples preincubated for the same period in the absence of inhibitor. Each point is the mean ± Standard Error of the ratio from triplicate determinations in a single experiment.

Figure 2. The Effects of FCE 26743 Concentrations on the Activities of Human Platelet Mitochondrial MAO-B



The enzyme preparation (0.2 mg/ml) was incubated with the indicated concentration of FCE 26743 for (□) 0 time or (●) 30 min. before activity was determined towards 20 μM PEA. radiochemically. Percentage inhibition was calculated with respect to samples preincubated for the same period in the absence of inhibitor. Each point is the mean ± Standard Error of the ratio from triplicate determinations in a single experiment.

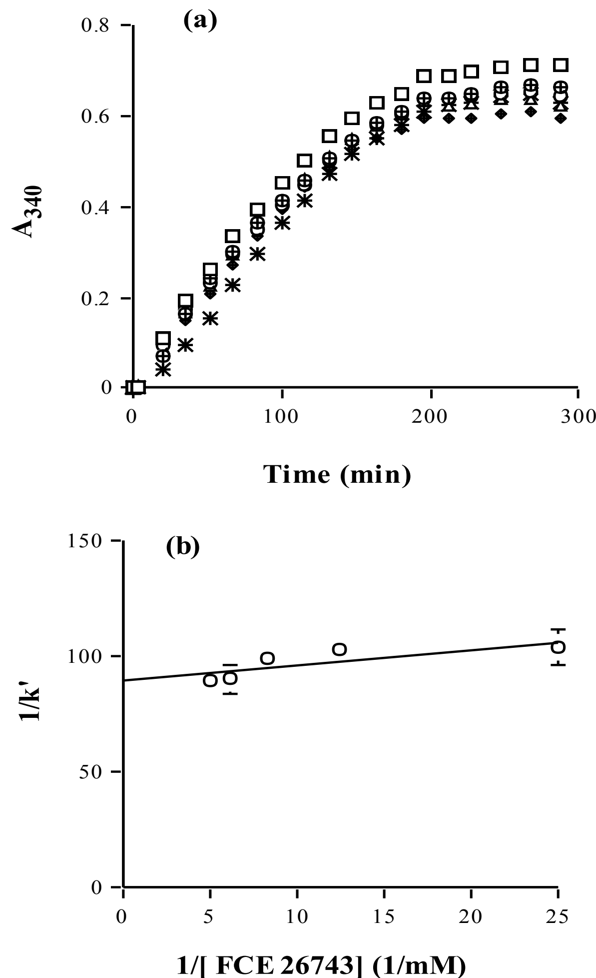
Figure 3. Kinetics of the Inhibition of Ox Liver Mitochondrial MAO-B by FCE 26743



a) The Inhibition of Ox Liver Mitochondrial MAO-B by FCE 26743.
Initial rates were measured spectrophotometrically at 250nm in the presence of the indicated concentrations of Benzylamine and in the presence of (■) 0, (△) 20 µM, (▲) 40 µM, (□ ○) 80 µM, (●) 100 µM,(□) 150 µM FCE 26743. Each point is the mean of three separate experiments.

b) Determination of the K_i Value of FCE 26743 towards Ox Liver Mitochondrial MAO-B.
The dependence of the slopes obtained from the double reciprocal plots shown above on FCE 26743 concentration. The intercept of the extrapolated line on the FCE26743 concentration axis gives a value of K_i.

Figure 4. Inhibition of Human Brain Mitochondrial MAO-A by FCE26743 at a Series of Different Concentrations



a. Time Courses of Inhibition of Human Brain Mitochondrial MAO-A by FCE26743 at a Series of Different Concentrations

The reactions of human brain mitochondria (98 μg/ml) with 5HT (at 37 °C and pH7.2) in the presence of: 0 (✱), 40(◆), 80 (△), 120 (○), 160 (⊕) and 200 (□) μM FCE 26743 were monitored spectrophotometrically at 340nm. The points shown are the results from 6 representative experiments.

b. Determination of the Kinetic Parameter K_i and k_{+2} for the Effects of FCE 26743 on Human brain Mitochondrial MAO-A

A double reciprocal plot of the dependence of the apparent rate constants obtained from the time courses of inhibition as shown above on the inhibitor concentration. The slope and the intercept of this line give the values of K_i and k_{+2} respectively. Each point represents the mean of three separate experiments.