



Human monoamine oxidase enzyme inhibition by coffee and β -carbolines norharman and harman isolated from coffee

Tomas Herraiz*, Carolina Chaparro

Spanish Council for Scientific Research CSIC Instituto de Fermentaciones Industriales, Juan de la Cierva, 3, 28006 Madrid, Spain

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Abstract

Monoamine oxidase (MAO) is a mitochondrial outer-membrane flavoenzyme involved in brain and peripheral oxidative catabolism of neurotransmitters and xenobiotic amines, including neurotoxic amines, and a well-known target for antidepressant and neuroprotective drugs. Recent epidemiological studies have consistently shown that coffee drinkers have an apparently lower incidence of Parkinson's disease (PD), suggesting that coffee might somehow act as a purported neuroprotectant. In this paper, "ready to drink" coffee brews exhibited inhibitory properties on recombinant human MAO A and B isozymes catalyzing the oxidative deamination of kynuramine, suggesting that coffee contains compounds acting as MAO inhibitors. MAO inhibition was reversible and competitive for MAO A and MAO B. Subsequently, the pyrido-indole (β -carboline) alkaloids, norharman and harman, were identified and isolated from MAO-inhibiting coffee, and were good inhibitors on MAO A (harman and norharman) and MAO B (norharman) isozymes. β -carbolines isolated from ready-to-drink coffee were competitive and reversible inhibitors and appeared up to 210 $\mu\text{g/L}$, confirming that coffee is the most important exogenous source of these alkaloids in addition to cigarette smoking. Inhibition of MAO enzymes by coffee and the presence of MAO inhibitors that are also neuroactive, such as β -carbolines and eventually others, might play a role in the neuroactive actions including a purported neuroprotection associated with coffee consumption.

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Introduction

Monoamine oxidase (MAO) is a flavin-adenosine-dinucleotide (FAD)-containing enzyme located at the outer membranes of mitochondria in the brain, liver, intestinal mucosa, and other organs and catalyzes the oxidative deamination of biogenic amines (neurotransmitters, vasoactive and xenobiotic amines), including dopamine, serotonin, norepinephrine, tyramine, tryptamine and MPTP (*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) neurotoxin. MAO appears as two isozymes, MAO-A and MAO-B, distinguished by their differences in substrate and inhibitor selectivities (Johnston, 1968; Kalgutkar et al., 2001; Tipton, 1994). MAO A preferentially catalyzes the oxidation of serotonin and norepinephrine and is inhibited by clorgyline, whereas MAO B selectively catalyzes the oxidation of

phenylethylamine and benzylamine and is inhibited by (R)-deprenyl. Tyramine, dopamine, and tryptamine appear to be substrates for both enzymes. MAO plays a significant physiological role in the central nervous system and peripheral organs (O'Carroll et al., 1983; Shih et al., 1999). Abnormal activity of MAO-B is implicated in neurological disorders such as Parkinson's and Alzheimer's diseases, whereas MAO-A plays an important role in psychiatric conditions such as depression (Yamada and Yasuhara, 2004). Identification of MAO inhibitors is of great interest in drug discovery (Kalgutkar et al., 2001; Yan et al., 2004). MAO A inhibitors have been used as antidepressants (Nolen et al., 1993), and MAO B inhibitors in the treatment and prevention of Parkinson's disease (Ben-Shlomo and Bhatia, 2004). The oxidation of biogenic amines by MAO results in the production of hydrogen peroxide and aldehydes which may represent a risk factor for cell oxidative injury (Cohen et al., 1997; Hauptmann et al., 1996; Vindis et al., 2000; Youdim and Lavie, 1994). In the brain, MAO activates the dopaminergic proneur-

* Corresponding author. Tel.: +34 91 5622900; fax: +34 91 5644853.
E-mail address: therraiz@ifi.csic.es (T. Herraiz).

otoxin MPTP to the active neurotoxic metabolite MPP⁺ (*N*-methyl-4-phenylpyridinium) producing PD (Langston et al., 1984). Protection against Parkinsonism by MAO inhibitors could result from reduced toxin activation as well as reduced production of hydrogen peroxide, ammonia and aldehyde species.

MAO activity is reduced in smokers who exhibit up to a 28% lower brain MAO A and a 40% lower MAO B compared to nonsmokers (Fowler et al., 1996a,b, 2003; Orelan et al., 1981). This finding is relevant since MAO inhibition in smokers may be linked with the addictive properties of cigarettes and depression [Berlin et al., 1997; Glassman et al., 2001], and also with the lowest incidence of Parkinson's disease among smokers (Fowler et al., 2003; Castagnoli and Murugesan, 2004). As occur with cigarette smokers, several epidemiological studies have found an apparent strong negative correlation between coffee consumption and the incidence of Parkinson's disease (up to a 30% reduction in risk), suggesting that regular coffee drinking might somehow protect against the development of neurodegenerative diseases (Ascherio et al., 2001; Benedetti et al., 2000; Hernan et al., 2002; Tanner et al., 2002; Ragonese et al., 2003; Ross et al., 2000). Coffee is a popular beverage largely enjoyed and consumed for its sensory and stimulant properties, and the object of interest in human health (Quinlan et al., 2000; Woodward and Tunstall-Pedoe, 1999). It contains active constituents, including caffeine, a strong stimulant that enhances alertness and sustained attention acting as an adenosine antagonist (Fredholm et al., 1999). Coffee enhances alertness, concentration, mental and physical performance, and may cause a temporary rise in blood pressure. However, the causes for a purported neuroprotective effect of coffee remain unknown and further research is actually needed to confirm this action. This effect could be related to adenosine A_{2A} receptor antagonism (caffeine effect) or to MAO inhibition (Chen et al., 2002). Neuroprotection is often associated with MAO inhibition and an eventual neuroprotection in tobacco smokers may be linked to MAO inhibition (Castagnoli and Murugesan, 2004; Fowler et al., 2003; Herraiz and Chaparro, 2005). As far as we know, there is no previous report on MAO inhibition produced by coffee or in coffee drinkers in contrast to cigarette smoke and smokers (Fowler et al., 2003; Hauptmann and Shih, 2001; Herraiz and Chaparro, 2005; Khalil et al., 2000; Méndez-Alvarez et al., 1997; Orelan et al., 1981; Rommelspacher et al., 2002; Yu and Boulton, 1987). In a recent work we confirmed that cigarette smoke exerted potent MAO inhibition and identified β -carboline alkaloids from smoke as potent MAO inhibitors (Herraiz and Chaparro, 2005). The present research was aimed to investigate whether coffee brews may inhibit recombinant human MAO enzymes, and further study the occurrence of components in coffee acting as MAO inhibitors. As a result, it is shown that ready-to-drink coffee inhibited the activity of MAO-deaminating kynuramine, suggesting that MAO inhibitors likely occur in coffee. Subsequently, two β -carboline alkaloids (norharman and harman) acting as good and reversible inhibitors were identified and isolated from coffee brews. These β -carbolines

may contribute to an eventual MAO-reduced activity in regular coffee drinkers.

Materials and methods

Recombinant human monoamine oxidase A and B were obtained from Gentest BD biosciences (Woburn, MA, USA). Enzymes were expressed from MAO-A and MAO B cDNA using a baculovirus expression system, and prepared as membrane protein fractions. Membrane fractions without enzymes were used as controls. Clorgyline, *R*-deprenyl, kynuramine, 4-hydroxyquinoline, norharman and harman were purchased from Sigma. HPLC grade acetonitrile, methanol and dimethylsulfoxide (DMSO) were from Scharlau (Spain) and dichloromethane from Merck.

Several samples of ready to drink coffee brews were obtained as follows: a) instant coffee prepared from commercial instant ground coffee by dissolving 4 g in hot water (40 mL); b) commercial ready to drink espresso coffee (80 mL) was obtained from a cafeteria, and c) regular coffee prepared in a household coffee-maker with ground coffee (20 g) and hot water (160 mL). Coffee brews were conveniently diluted and aliquots were used for enzymatic studies, HPLC analysis, and β -carboline isolation.

Monoamine oxidase assay (MAO A and B)

Membrane protein fractions containing MAO-A or MAO-B were diluted to the desired concentrations in 100 mM potassium phosphate buffer (pH 7.4). A 0.2 mL reaction mixture containing 0.01 mg/mL protein and 0.25 mM kynuramine in 100 mM potassium phosphate (pH 7.4) was incubated at 37 °C for 40 min. After incubation the reaction was stopped by the addition of 2N NaOH (75 μ L), followed by the addition of 70% perchloric acid (25 μ L), and the sample centrifuged (10,000 \times g) for 5 min. The supernatant (20 μ L) was injected into the HPLC and deamination products of kynuramine formed during the enzymatic reaction were determined by RP-HPLC-DAD and fluorescence detection. Under these conditions, kynuramine deaminated by MAO spontaneously cyclizes to give 4-hydroxyquinoline, that was determined at 320 nm. A response curve of area vs. concentration was constructed to calculate the concentration of 4-hydroxyquinoline. To perform inhibition assays, aliquots of coffee brews, or instead isolated β -carbolines from coffee brews were added to reaction mixture containing kynuramine (0.25 mM) in 100 mM potassium phosphate buffer (pH 7.4), and an appropriate amount of MAO enzyme (A or B) was added to initiate the reaction. The mechanism of MAO inhibition was assessed by analyzing the corresponding double reciprocal Lineweaver-Burk plots and kinetics values of the Michaelis constant (K_m) and the maximum velocity (V_{max}) were also obtained from non-linear regression analysis (velocity vs. concentration). IC_{50} values were calculated by adjusting the experimental data (% inhibition vs concentration of inhibitor) to non-linear regression curves.

Determination of MAO binding reversibility

A previously reported procedure was followed (Yan et al., 2004). Membrane proteins containing MAO-A or MAO B (0.2 mg/mL) in 100 mM phosphate buffer (pH 7.4) were incubated at 37 °C for 40 min with test compound (5–20 μ M) or coffee brews (1/3 diluted instant coffee, 40 μ L–80% MAO inhibition). Reaction mixtures were centrifuged (15,000 \times g) for 15 min to pellet membrane proteins, washed twice with 100 mM phosphate buffer (pH 7.4) and resuspended in 100 mM phosphate buffer (pH 7.4)+10% DMSO (0.1 mL). An aliquot was used to measure MAO activity and this enzymatic activity compared with controls.

RP-HPLC chromatographic analysis

The analysis of the kynuramine deamination product 4-hydroxyquinoline was performed by RP-HPLC with *uv-DAD* and fluorescence detection using an HPLC 1050 (Hewlett Packard) provided with a Diode Array Detector (DAD) and a 1046A-fluorescence detector. A 150 mm \times 3.9 mm, 4 μ m, Nova-pak C18 column (Waters, Milford, MA, USA) was used for chromatographic separation. Chromatographic conditions were: 50 mM ammonium phosphate buffer (pH 3) (buffer A) and 20% of A in acetonitrile (buffer B). Gradient programmed from 0% (100% A) to 32% B in 8 min, and 90% B at 15 min. The flow rate was 1 mL/min, the column temperature was 40 °C and the injection volume was 20 μ L. Absorbance detection was set at 320 nm and the fluorescence at 300 nm (excitation) and 350 nm (emission). Identification of 4-hydroxyquinoline was done by UV and fluorescence spectra, and co-injection with authentic standards. Data from absorbance (320 nm) detection were generally used for quantitation, although fluorescence was used in MAO inhibition experiments with coffee brews to avoid interferences.

Isolation of β -carbolines from coffee and HPLC-ESI-mass spectrometry

The analysis, and isolation of β -carbolines in coffee was performed using Solid Phase Extraction and RP-HPLC as previously (Herraiz, 2002). β -carbolines norharman and harman were determined by absorbance at 254 nm and fluorescence detection at 300 nm (excitation) and 433 nm (emission). Quantitative analysis was carried out using calibration curves (area against concentration) constructed with authentic standards of β -carbolines. To isolate β -carbolines, an aliquot (20–30 mL) of diluted and acidified coffee brew was passed through propylsulfonic acid (PRS)-derivatized silica columns (Herraiz, 2002). The eluates were pooled and extracted with dichloromethane (30 mL), that was subsequently evaporated, and added with 100 mM phosphate buffer (pH 7.4) containing 30% DMSO (600 μ L). Successive RP-HPLC injections (20 μ L) were carried out into RP-HPLC as mentioned above, and the chromatographic peaks corresponding to β -carbolines norharman and harman collected. After removing the HPLC-acetonitrile in rotoevaporator, β -carbolines from coffee were

extracted again at pH 9 with dichloromethane, concentrated and resuspended in 100 mM phosphate buffer (pH 7.4) containing 30% DMSO (100 μ L). Aliquots of these extracts were used to study the MAO inhibition activity of β -carbolines isolated from coffee. Identification of β -carbolines from coffee was performed by HPLC-MS. For that, coffee extracts (100 μ L) coming from PRS columns and solvent-solvent extraction were analyzed in a 2.1 \times 150 mm Zorbax SB-C18, 5 μ m column (Agilent Technologies), by using an HPLC-MSD series 1100 (Hewlett-Packard) (electrospray-positive ion mode). Eluent A: formic acid (0.5%); B: 0.5% formic acid in acetonitrile; 80% B 30 min, flow rate 0.25 mL/min. T: 40 °C, Mass range 50–700 amu. Cone voltage 100 V.

Results

Enzymatic activity of protein fractions containing human MAO A and MAO B and its inhibition by ready to drink coffee was studied with several diluted coffee brews. MAO A and MAO B enzymes were inhibited by the different coffee brews assayed (i.e. instant, espresso and regular brewed coffee), with instant coffee showing the highest degree of inhibition (Fig. 1). Enzymatic activity of MAO A and B isozymes were

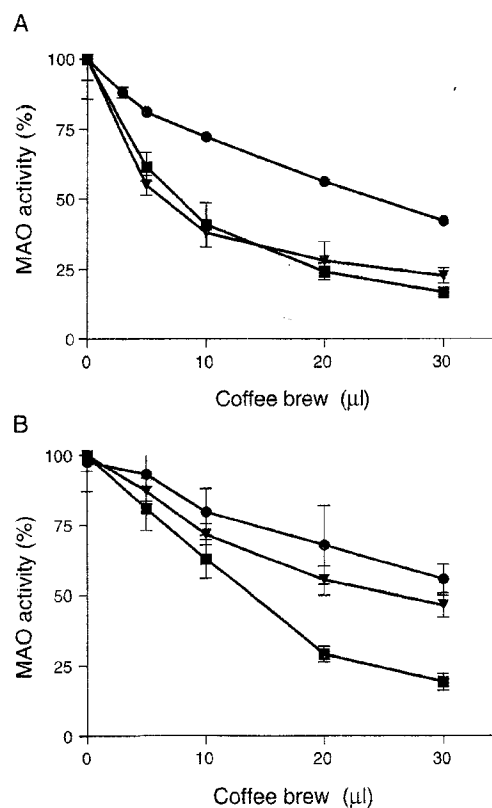


Fig 1 Activity of monoamine oxidase MAO A (A) and MAO B (B) enzymes over kynuramine deamination in presence of ready to drink coffee brews from instant coffee (1/3-diluted) (■), espresso coffee (1/2-diluted) (●), and regular coffee (▼). Coffee brews were prepared as indicated in Experimental. Data are from duplicate experiments

determined under varying kynuramine concentrations in control assays and in presence of diluted instant coffee brews, and the double reciprocal plots are given in Fig. 2. The inhibition kinetics of coffee over MAO-kynuramine deamination resulted competitive for MAO A and MAO B. Inhibition produced by coffee brews (instant coffee) was reversible ($81 \pm 7\%$ for MAO A and $99 \pm 0.8\%$ for MAO B), as suggested from the activity recovered following incubation of enzyme with coffee compared with controls under the same conditions.

Coffee brew exhibited inhibitory properties on the deamination of kynuramine by MAO enzymes (A and B isozymes), and therefore it may contain specific compounds that could act as MAO inhibitors when absorbed and/or accumulated in coffee drinkers. To isolate some of those compounds, instant coffee with a high degree of MAO inhibition (Fig. 1), was subjected to solid phase extraction using propylsulfonic-PRS columns. Chromatographic analysis evidenced the presence of two β -carbolines identified by co-injection with standards, UV-VIS spectra and HPLC-Mass Spectrometry as the pyridoindole norharman (tr 7.6 min, UV maxima at 248, 302 and 370 nm, and mass spectra with m/z (M+H)⁺ at 169) and harman (tr 8.1 min, UV max at 248, 302 and 365 nm, and m/z (M+H)⁺ at 183). β -carbolines from instant coffee were separately isolated

following successive HPLC injections by collecting the chromatographic peaks and then used to study the inhibition kinetics under varying concentration of kynuramine (Fig. 3). Inhibition of MAO A by both norharman and harman was a competitive type of inhibition and higher for harman than for norharman. Inhibition of human MAO-B showed that norharman but not harman produced a significant competitive inhibition. Activities of MAO A and B were fully restored following incubation with β -carbolines isolated from coffee, showing that they are reversible inhibitors (results not shown). In the same assay *R*-deprenyl and clorgyline at 250 nM were irreversible inhibitors of MAO B and A, respectively. We have calculated inhibition values (IC_{50}) of 0.34 μ M (harman) and 6.5 μ M (norharman) over MAO A, and 4.7 μ M (norharman) over MAO B (Herraiz and Chaparro, 2005). Then, the inhibition of MAO A and B isozymes produced by norharman and harman isolated from instant coffee was in good agreement with the concentration of these compounds actually used into the assays (Fig. 3). Thus, harman isolated from coffee (0.3 μ M into the assay) gave a 44% inhibition over MAO A, whereas norharman (2.7 μ M into the assay) gave a 30% inhibition over MAO A and a 38% over MAO B, using kynuramine (250 μ M) as a substrate.

Finally, we measured the concentration of β -carbolines in coffee brews ($n=4$) and obtained the following results (mean \pm SEM): 144 ± 17 μ g/L (norharman) and 36 ± 4.5 μ g/L (harman) in espresso; 3.5 ± 1.4 μ g/g of instant-ground coffee (norharman), and 0.73 ± 0.34 μ g/g coffee (harman) in instant coffee brews; and 1.8 ± 0.48 μ g/g of ground coffee (norharman) and 0.4 ± 0.1 μ g/g (harman) in ground-brewed regular coffee. These results clearly indicate that concentration of β -carboline alkaloids acting as MAO inhibitors in ready-to-drink coffee brews varied substantially with the type of coffee brew and mainly with the amount of ground coffee used to make brewed coffee.

Discussion

The primary role of MAO isozymes lies in the metabolism of amines, regulating neurotransmitter levels and intracellular amine stores. Within neurons, MAO appears to regulate the levels of neurotransmitters released upon synaptic firing. In the gastrointestinal tract, the circulatory system and the liver, MAO may serve a protective function by regulating the levels of exogenous dietary amines exerting potent vasopressor effects. In fact, the first classic use of MAO inhibitors as antidepressant was limited because they produced hypertensive crisis when the patients consumed tyramine-containing foods (the so-called "cheese effect") (Davies et al., 1978, Youdim and Weinstock, 2004). MAO metabolizes toxic xenobiotic amines such as MPTP (*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a dopaminergic neurotoxin known to produce Parkinson's disease (Langston et al., 1984). The oxidation of biogenic amines by MAO results in the production of potentially toxic hydrogen peroxide, ammonia, and aldehydes that represent a risk factor for cell oxidative injury (Hauptmann et al., 1996; Vindis et al., 2000). Protection against Parkinsonism and neurodegeneration by MAO inhibitors could result from a reduced toxin activation

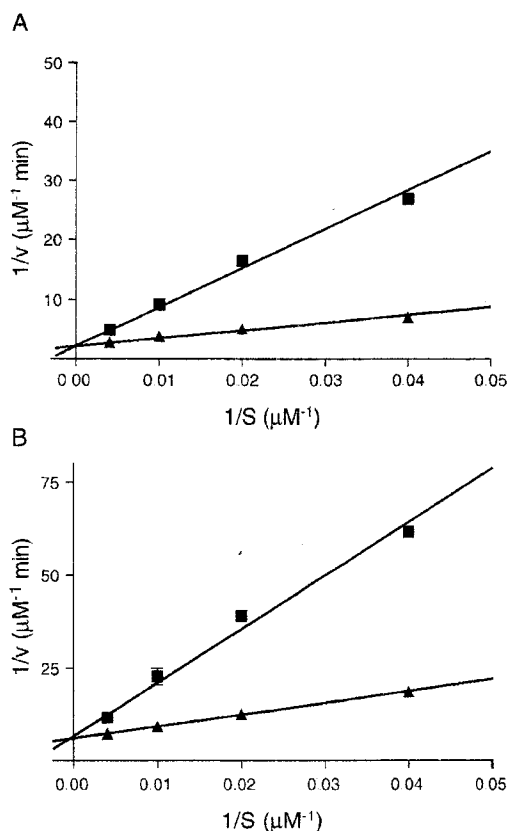


Fig. 2 Double reciprocal Lineweaver-Burk plots of human MAO A (A) and MAO B (B) kinetics in absence (control) (▲) or in presence of coffee brew (1/3-diluted instant coffee) (■) MAO A (5 μ L of coffee), and MAO B (10 μ L of coffee). Values obtained at least from duplicate experiments.

as well as reduced production of hydrogen peroxide, ammonia and aldehyde species (Cohen et al., 1997; Di Monte et al., 1997; Heikkila et al., 1984). Those biological implications of MAO are of high pharmacological interest and this enzyme is a known target for antidepressant drugs (MAO A inhibitors), and for drugs used in neurological disorders and diseases (MAO B inhibitors).

Results presented above show that “ready to drink” instant, espresso and regular coffee inhibited MAO enzymes. Because of its possible interest, these results deserve further consideration. However, as the inhibition data come from *in vitro* experiments obtained with a complex mixture as it is coffee brew, the conclusions drawn must be taken with caution and further studies are needed to fully demonstrate a possible link between regular coffee drinking and an effective reduction of MAO. Eventually, this might be accomplished as successfully done in smokers by Fowler et al. (1996a,b, 2003) using PET (positron emission tomography) studies. Interestingly, an eventual MAO reduction in regular coffee drinkers could allow further speculation on the purported neuroprotective effects of coffee (i.e. an apparent lower risk for developing Parkinson’s and neurodegenerative diseases in coffee drinkers), as suggested from epidemiological studies (Ascherio et al., 2001; Ross et al., 2000). Inhibition of MAO by coffee could spare neurotransmitters (dopamine), limit the activation of eventual proneurotoxins and reduce H_2O_2 and aldehydes, contributing to oxidative stress. On the other hand, coffee

consumption might produce peripheral inhibition of MAO that could eventually affect the metabolism of exogenous and dietary amines.

Coffee may contain compounds acting as inhibitors of MAO A and B isozymes. In this regard, two relevant β -carboline alkaloids (norharman and harman) were isolated from MAO-inhibiting coffee and acted as good and reversible MAO A (harman and norharman) and B (norharman) inhibitors. β -carbolines are pyrido-indole alkaloids produced through a Pictet–Spengler condensation from indolethylamines and carbonylic compounds. They occur and accumulate in biological tissues (Airaksinen and Kari, 1981; Fekkes and Bode, 1993; Matsubara et al., 1998; May et al., 1994; Yu et al., 2003a), and are also environmental and/or dietary xenobiotics (Herraiz, 2002, 2004a,b; Herraiz and Galisteo, 2002, 2003). β -Carbolines exert a wide spectrum of pharmacological and psychopharmacological effects, including antidepressant-like effects (Aricioglu and Altunbas, 2003; Robinson et al., 2003; Ruiz-Durantez et al., 2001). They alter the concentrations of brain neurotransmitters by interaction with serotonin, benzodiazepine, opioid, and imidazoline receptors, and MAO and cytochrome P450 enzymes (Adell et al., 1996; Airaksinen and Kari, 1981; Baum et al., 1996; Ergene and Schoener, 1993; Husbands et al., 2001; Kawanishi et al., 1994; Kim et al., 1997; Pawlik and Rommelspacher, 1988; Rommelspacher et al., 2002; Yu et al., 2003a,b). The presence of these compounds in the human brain has been interpreted as possible mediators in

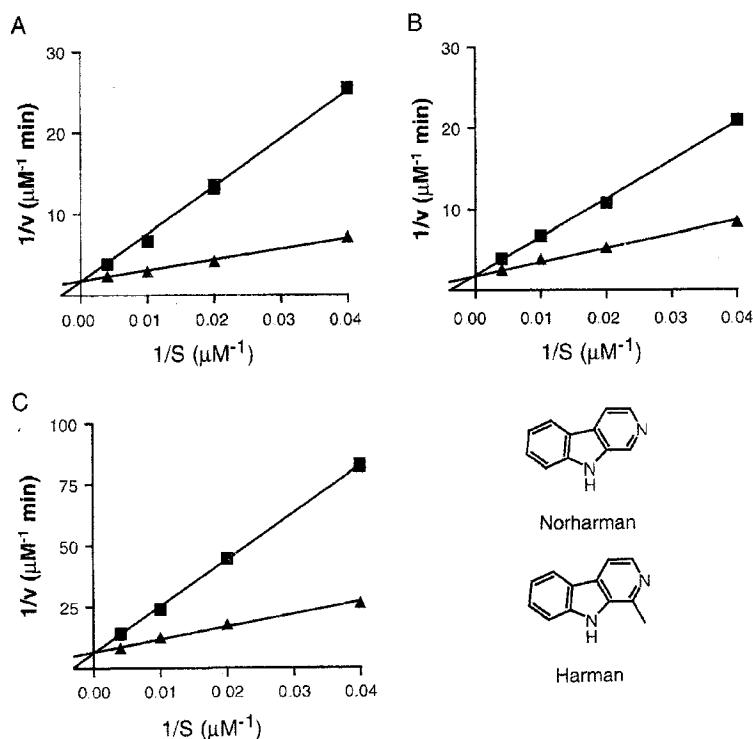


Fig. 3. Double reciprocal plots of human monoamine oxidase activity in presence of β -carbolines (■) isolated from coffee, versus controls (▲) (A) MAO A in presence of harman; (B) MAO A in presence norharman, and (C) MAO B in presence of norharman. The amount of β -carbolines isolated from instant coffee by SPE-HPLC and included into assay was calculated as 0.34 and 2.7 μM for harman and norharman, respectively. Values are at least from duplicate experiments.

alcoholism and addiction (Myers, 1989; Rommelspacher and Schmidt, 1985). On the basis of the amount of β -carbolines measured in MAO-inhibiting coffee, other compounds present in coffee should also account for MAO inhibition. Nevertheless, β -carbolines are readily absorbed and distributed in tissues and the brain, in which they may accumulate and reach local concentrations higher than plasma (Breyer-Pfaff et al., 1996; Kawanishi et al., 1994; Matsubara et al., 1998; Östergren et al., 2004; Rommelspacher et al., 2002; Spijkerman et al., 2002). As coffee is the main exogenous source of these alkaloids in addition to smoking (Herraiz, 2002, 2004b), accumulation of these compounds in regular coffee drinkers, might locally affect MAO metabolism of both exogenous amines and neuroamines. Additionally, β -Carbolines from coffee might exert potential pharmacological actions on other targets such as serotonin, opioid, and imidazoline receptors linked to MAO proteins (Robinson et al., 2003; Tesson et al., 1995). On the other hand, a different research perspective currently focuses on β -carbolines as potential endogenous and/or environmental proneurotoxins involved in Parkinson's disease (Gearhart et al., 2000; Matsubara et al., 1998). This is based on the fact that under bioactivation by *N*-methyltransferases, β -carbolines afford neurotoxic *N*-methyl- β -carbolinium cations structurally similar to MPP⁺ that is produced from MPTP with the participation of MAO (Collins and Neafsey, 1985; Langston et al., 1984). In this regard, the relatively highest exposure to β -carbolines from coffee and smoke (Herraiz, 2002, 2004b) which are epidemiologically related to a lowest incidence of PD, does not appear to support the assumption that dietary or environmental norharman and harman, by themselves, are direct causative agents in neurodegenerative (Parkinson's) diseases. However, they may play a role as MAO inhibitors, as reported here. Further studies are needed to know the complete implications of dietary, environmental and endogenous β -carbolines in human health.

In conclusion, ready to drink coffee brews inhibited MAO A and MAO B kynuramine deamination under a competitive and reversible type of inhibition, suggesting that coffee may contain active MAO inhibitors. In this regard further studies are needed to confirm whether MAO inhibition is produced in regular coffee drinkers, and most importantly, whether this may be related with an eventual neuroprotective action of coffee against PD. Nevertheless, two relevant β -carboline alkaloids (i.e. norharman and harman), were isolated from ready to drink coffee and acted as good competitive and reversible inhibitors of MAO A (norharman and harman) and MAO B (norharman). β -carbolines might be contributors to MAO-inhibitory actions of coffee but might also exert other neuropharmacological and behavioral effects. The same two compounds have been recently reported as contributors to MAO inhibition from cigarette smoke (Herraiz and Chaparro, 2005).

Acknowledgements

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