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CHROMATOGRAPHIC STUDIES ON THE BINDING, ACTION AND METABOLISM OF (−)-DEPRENYL

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SUMMARY

Serum binding, the effect on striatal dopamine release and the metabolism of (−)-deprenyl [N-methyl-N-propargyl(2-phenyl-1-methyl)ethylammonium chloride], TZ-650 [N-methyl-N-propargyl(2-phenyl)ethylammonium chloride] and J-508 [N-methyl-N-propargyl(indanyl)ammonium chloride] were investigated using various chromatographic methods. A strong interaction between (−)-deprenyl and macroglobulins was found. Deprenyl enhanced the dopamine release from striatal slices of the rat brain and also inhibited the dopamine–DOPAC conversion. Deprenyl analogues showed either smaller or no effect. Hydroxylation of (−)-deprenyl takes place in the *para* position, in addition to the usual oxidative N-dealkylations, which are known from various metabolic studies on N-substituted phenylalkylamines.

INTRODUCTION

(−)-Deprenyl, also called Eldepryl®, Jumex®, Movergan® and Selegiline®, is a potent, irreversible and specific inhibitor of the monoamine oxidase B enzyme[1–4]. Its peculiar pharmacological properties and beneficial clinical effects in Parkinson's disease prompted investigations of its binding, action and metabolism and studies of structural analogues.

Johnston[5] reported that clorgyline preferentially inhibits the oxidative deamination of serotonin, while Knoll and Magyar[2] found that (−)-deprenyl is a selective inhibitor of the deamination of benzylamine. These facts furnished indirect evidence that there are two different types of monoamine oxidase enzyme, monoamine oxidase A (MAO-A) and monoamine oxidase B (MAO-B). MAO-A is clorgyline-sensitive, whereas MAO-B is clorgyline-insensitive and deprenyl-sensitive. These inhibitors belong to the so-called suicide inhibitor group, meaning that while the enzyme splits this

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substrate, it suffers covalent binding, which results in an irreversible inhibition. Salach *et al.*[6] provided direct evidence that these enzyme inhibitors covalently interact with the flavin-active site of the enzyme. Ekstedt *et al.*[7] and Birkmayer and Yahr[8] showed that, in spite of repeated administration of (−)-deprenyl, a fairly selective inhibition pattern was maintained in the brain of rats and liver of human subjects.

(−)-Deprenyl is a monoamine oxidase inhibitor lacking the so-called cheese effect. It is not only free of this side-effect of MAO inhibitors but also, as Knoll *et al.*[9] have shown, it inhibits the uptake of tyramine. Similar observations in normal and Parkinsonian volunteers showed no adverse pressor reaction after a challenge with an oral dose of tyramine in amounts considerably larger than those likely to be encountered in a normal diet. These properties of (−)-deprenyl allow its safe administration to Parkinsonian patients on levodopa treatment[3].

EXPERIMENTAL

Materials

(−)-Deprenyl [N-methyl-N-propargyl(2-phenyl-1-methyl)ethylammonium chloride], *o*-methyldeprenyl, *m*-methyldeprenyl, *p*-methyldeprenyl, TZ-650 [N-methyl-N-propargyl(2-phenyl)ethylammonium chloride] and J-508 [N-methyl-N-propargyl(indanyl)ammonium chloride] were kindly supplied by Chinoin (Budapest, Hungary). In some experiments, (−)-deprenyl was labelled in position 2 of the (2-phenyl-1-methyl)ethyl moiety (Fig. 1).

3,4-Dihydroxybenzylamine (DHBA) (Calbiochem, San Diego, CA, U.S.A), dopamine hydrochloride (Serva, Heidelberg, F.R.G.), 3,4-dihydroxyphenylacetic acid (DOPAC) (Fluka, Buchs, Switzerland), alumina, Brockmann Grade II, neutral, and LiChrosorb C_{18}, 5 μm (Merck, Darmstadt, F.R.G.) and sodium octanesulphonate (Aldrich, Milwauke, WI, U.S.A.) were used. All the other chemicals and solvents were of the highest commercially available quality.

Sephadex G-15 and Sephadex G-200 Fine (Pharmacia, Uppsala, Sweden) were used for size exclusion chromatography. They were packed into glass columns 90 or 60 cm × 2.5 or 5.0 cm I.D. For elution 0.05 *M* ammonium acetate solution was used, and 10-ml fractions of the eluate were collected with an automatic fraction collector (Labor-MIM, Budapest, Hungary). Experiments were carried out with parts of a ReCyChrom system (LKB, Bromma, Sweden) including a cold box adjusted to 4°C, a peristaltic pump, a Uvicord UV detector set at 254 nm and a recorder.

For thin-layer chromatography (TLC), all-glass developing chambers (Desaga, Heidelberg, F.R.G.) were used. The spots were revealed with ninhydrin reagent and by autoradiography by previously published methods[10].

For high-performance liquid chromatographic (HPLC) experiments a Liquopump 312 solvent-delivery system with injection valve fitted to a 20-μl loop was used (Labor-MIM). The 250 × 4.6 mm I.D. column (Labor-MIM) was packed with Li-

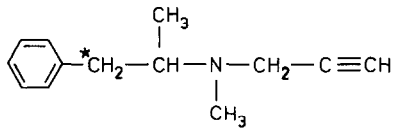

Fig. 1. Structure of deprenyl. The asterisk indicates the position of ^{14}C labelling.

Chrosorb C_{18}. A TL-3 thin-layer electrochemical detector cell with a paraffin oil-based carbon paste working electrode was purchased from Bioanalytical Systems (West Lafayette, IN, U.S.A.). It was used at 0.7 V and connected to an electronic controller, developed by the Biomedical Engineering Workshop of the Department of Pharmacology, Semmelweis University of Medicine (Budapest, Hungary). The signal from the amplifier was observed on an OH 814/1 potentiometric recorder (Radelkis, Budapest, Hungary). The mobile phase was a triethylamine phosphate buffer (pH 3). This buffer also contained 75 mg/l sodium octanesulphonate as an ion-pairing agent and 18 mg/l of Na_2EDTA to reduce the spike activity of the detector. Finally, 6% (v/v) acetonitrile was added to the mobile phase, which was degassed under vacuum and sonication. The internal standard was 3,4-dihydroxybenzylamine. Results were calculated from the peak-height ratios of dopamine to internal standard and DOPAC to internal standard; the dopamine release from rat brain striata was expressed in pmol/g · min. For statistical evaluations Student's *t*-test for two means was applied. The significance level was set at $P < 0.05$.

An HP-5985B gas chromatograph–mass spectrometer (Hewlett-Packard, Palo Alto, CA, U.S.A.) was connected to an HP-2648A data analyser. Separation was achieved by temperature programming from 100 to 320°C at 5°C/min[11]. Glass capillary columns (25 × 0.25 mm I.D.) were used. The stationary phase was 20% diphenyl- and 80% dimethylpolysiloxane (SPB 20) (Supelco, Bellefonte, PA, U.S.A.) and the flow-rate of the helium carrier gas was 1 ml/min. The gas chromatograms and the mass spectra were recorded with a thermal printer.

Methods

To investigate protein–deprenyl adducts, human serum (obtained from the National Institute for Blood Transfusion, Budapest, Hungary) was incubated with radiolabelled deprenyl for 2 h. The sample was immediately separated by gel chromatography on Sephadex G-15. Protein–deprenyl adducts, eluted at the void volume of the column, were further separated on Sephadex G-200.

The study on dopamine release following chronic pretreatment was performed after three weeks of subcutaneous administration of (−)-deprenyl, TZ-650 or J-508 in one daily dose to rats. Twenty-four hours after the last injection, the rats were decapitated. Striata were immediately removed and halved according to the method of Glowinski and Iversen[12,13], then soaked in Krebs solution. Four striata were pooled in one organ bath, incubated for 1 h, and then the Krebs solution was replaced. Subsequently, the Krebs solution was changed twice at 10-min intervals. Finally, the Krebs solution was changed to one which contained 20 m*M* potassium chloride. The striata were exposed to the latter medium for 10 min in order to stimulate the transmitter release. Samples were collected after each 10-min incubation period. The samples were spiked with 40 ng of 3,4-dihydroxybenzylamine (internal standard) and prepurified on a 90-mg alumina microcolumn according to the method of Anton and Sayre[14]. The effluent from the microcolumn was injected into the HPLC column[13].

For the metabolic study, albino Wistar rats of both sexes, weighing 120–150 g, were used[14]. Solutions of (−)-deprenyl, TZ-650 and J-508 were injected subcutaneously in doses of 50 mg/kg. Urine was collected for 24 h, and either immediately extrated or kept at −40°C until analysed as follows. Urine was adjusted to pH 11

with 0.5 *M* sodium hydroxide solution and the basic metabolites were extracted with chloroform–ethyl acetate (3:1). The organic layer was re-extracted into the buffer at pH 1.5, and the extraction with chloroform–ethyl acetate (3:1) was repeated after the pH had been adjusted to 11.

RESULTS

In the study of the binding of (−)-deprenyl to serum proteins the radiolabelled drug was incubated with human serum for 2 h. The gel chromatograms are presented in Fig. 2. The radioactive peak at the void volume of the gel column indicates that there is definite binding between serum proteins and deprenyl (Fig. 2a). A substantial portion of deprenyl was bound to macroglobulins, while the albumins contained the smallest fraction of the radioactivity (Fig. 2b).

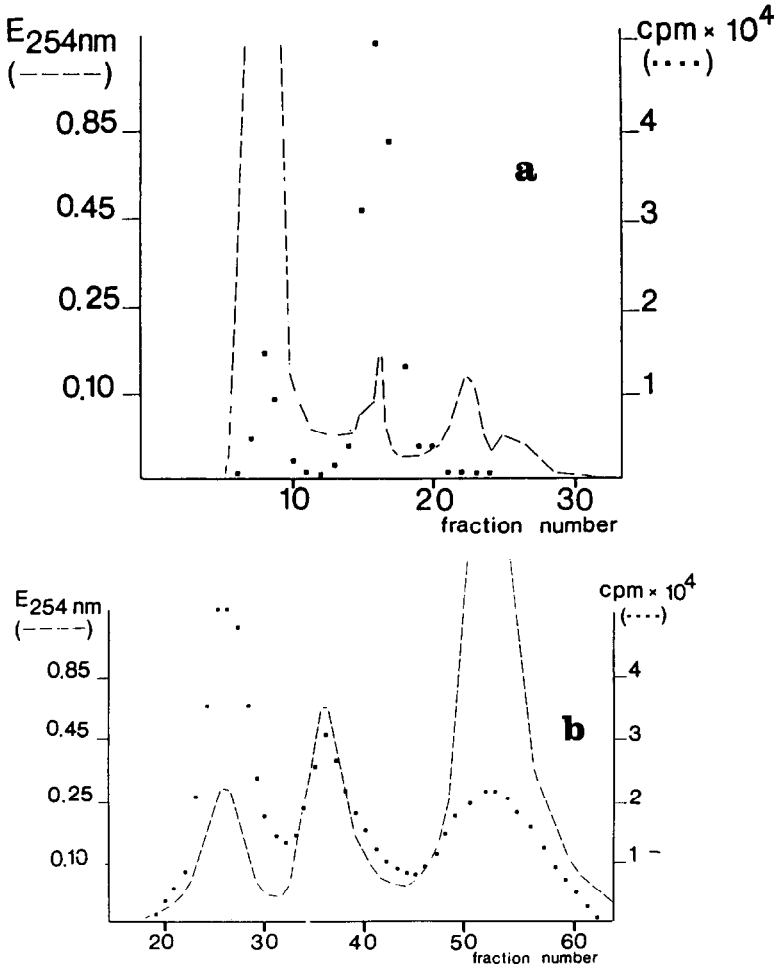

Fig. 2. (a) Gel chromatography of free and protein-bound deprenyl on Sephadex G-15. (b) Gel chromatographic separation of proteins containing deprenyl on Sephadex G-200. E_{254nm} = absorbance at 254 nm.

These experiments give direct evidence that the link between deprenyl and serum proteins is strong or irreversible, because deprenyl–protein adducts remained intact even after several hours of gel chromatography. Parallel experiments with reversibly binding substances did not show such a stability of drug–protein adducts in gel chromatography. Similar studies were performed using equilibrium dialysis cells.

Because the specific inhibitory effect of deprenyl on monoamine oxidase B does not explain its effectiveness as medication in Parkinsonism, its action was investigated on the release of dopamine from the striatum. The *in vitro* addition of (−)-deprenyl to the organ bath did not result in a noticeable change in dopamine release, except at very high concentrations, which were well above any therapeutic range. Three weeks of pretreatment with one daily dose of (−)-deprenyl increased the dopamine release and decreased its transformation to DOPAC in rats. Both the so-called resting release and the release induced by 20 m*M* of potassium were measured. It was found that 3 weeks of pretreatment with a 0.25 mg/kg daily dose of deprenyl increased the dopamine release to *ca.* five times the control level and inhibited the transformation of dopamine to DOPAC. A similar phenomenon was observed in the potassium-stimulated release where the increase was even more expressed (Table I). In experiments with the two structural analogues of (−)-deprenyl, TZ-605 was almost ineffective but J-508 was a stronger MAO inhibitior (less DOPAC was observed) (Table I).

In preliminary investigations of the metabolism of (−)-deprenyl, two-dimensional TLC was used. Development in the first dimension was performed by elution chromatography and displacement chromatography was applied in the second dimension (Fig. 3).

The metabolites of (−)-deprenyl, TX-650 and J-508 were identified by gas chromatography–mass spectrometry (GC–MS). The chromatograms obtained by total ion monitoring are presented in Figs. 4, 5 and 6, respectively. Neither deprenyl nor its

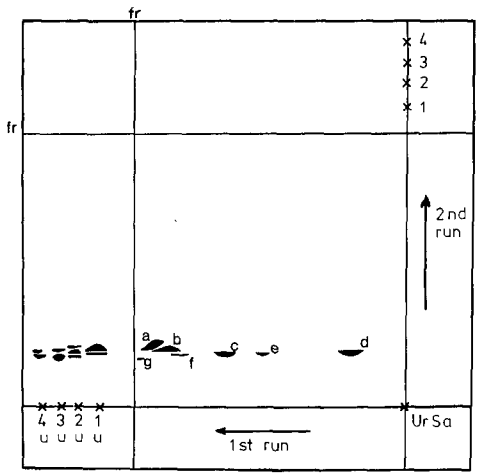

Fig. 3. Displacement TLC of deprenyl metabolites on silica plates with chloroform–methanol–water (29:20:4, v/v/v) and chloroform–triethanolamine (96:4, v/v) in the first and second dimensions, respectively. a = Deprenyl; b = propargylanara; c = methaphetamine; d = amphetamine; e, f and g are unidentified metabolites; u = urine sample; UrSa = urine sample for two-dimensional separation; fr = solvent front.

TABLE I

RELEASE OF DOPAMINE (DA) AND DOPAC UNDER VARIOUS CONDITIONS

	Release ± S.D. (pmol g^{-1} min^{-1})			
	Resting		*20 mM KCl stimulated*	
	DA	*DOPAC*	*DA*	*DOPAC*
Control	91 ± 7	258 ± 18	200 ± 15	291 ± 19
Deprenyl, 0.25 mg/kg	503 ± 20	72 ± 10	1451 ± 183	120 ± 36
J-508, 0.1 mg/kg	360 ± 27	18 ± 3	650 ± 26	19 ± 3
TZ-650, 0.1 mg/kg	151 ± 18	291 ± 23	272 ± 35	340 ± 36

metabolites gave a molecular ion, but only two main fragments. One of them originates from the benzyl part (the phenolic ring and the neighbouring methyl), which forms a cycloheptatrienium cation in the mass spectrometer, while the other fragment is the tri-, di- of monoalkyl-substituted nitrogen, *i.e.*, the remaining part of the compounds. Thus deprenyl gave fragments with mass numbers 91 + 96, while its de-

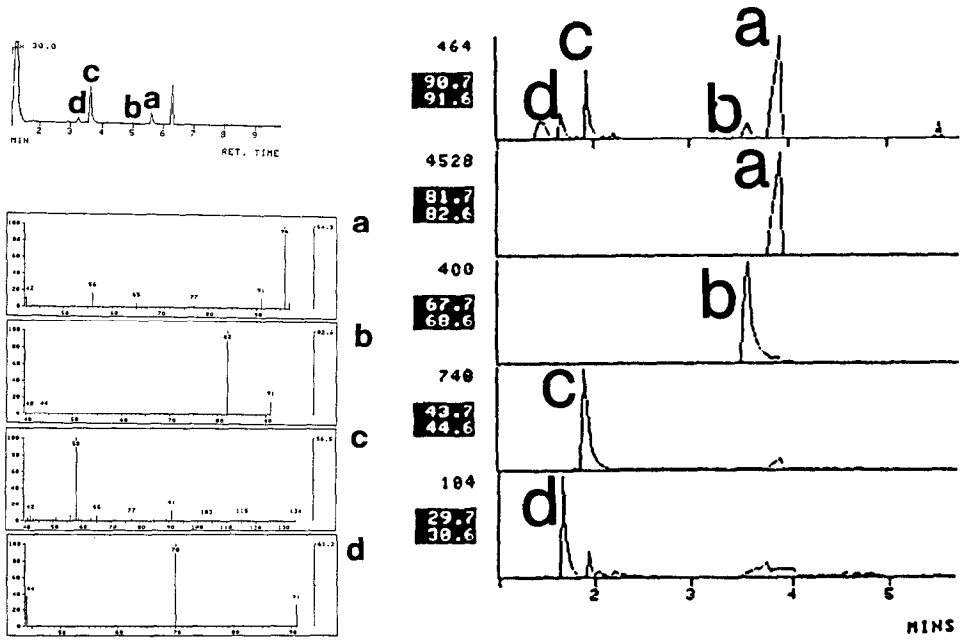

Fig. 4. GC–MS identification of deprenyl metabolites by selected ion monitoring. Peaks a, b, c and d are deprenyl, propargylanara, methamphetamine and amphetamine, respectively.

Fig. 5. GC–MS identification of TZ-650 metabolites by selected ion monitoring. Peaks a, b, c and d are the unaltered drug and its demethylated, depropargylated and didealkylated metabolites, respectively. The white numbers in the black boxes are the scanning ranges of the ion fragments (*e.g.*, 90.7 to 91.6 for *m/z* 91).

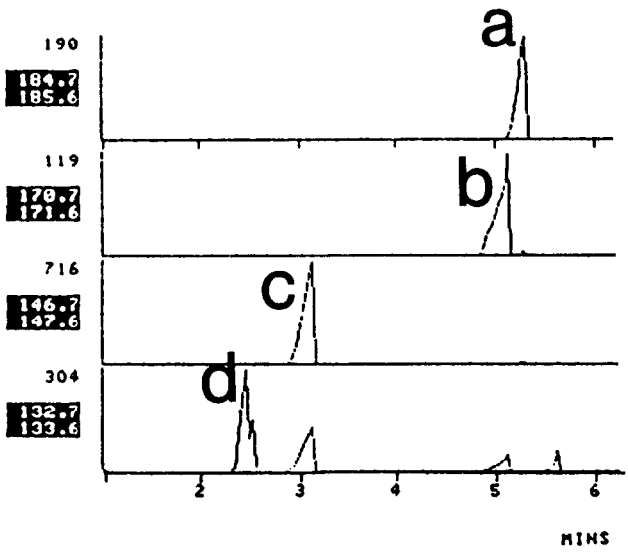

Fig. 6. GC–MS identification of J-508 metabolites using selected ion monitoring. Peaks a, b, c and d are the unaltered drug and its demethylated, depropargylated, and didealkylated metabolites, respectively. For the white numbers in the black boxes, see legend to Fig. 5.

methylated, depropargylated and demethylated/depropargylated products, propargylanara (its other name is propargylamphetamine), methamphetamine and amphetamine gave mass numbers 91 + 82, 91 + 58 and 91 + 44, respectively, indicating the respective losses of the nitrogen substituents. The results are shown in Fig. 4.

Figs. 5 and 6 show the results of the GC–MS investigations of the metabolism of TZ-650 and J-508. Mass fragment 91 characterizes the intact phenolic ring, whereas the ions with mass numbers 82, 68, 44 and 30 represent the intact side-chain of TZ-650 and its demethylated, depropargylated and demethylated/depropargylated derivatives, respectively.

With J-508, molecular ions with mass numbers 185, 171, 147 and 133 indicate the presence of J-508 and its demethylated, depropargylated and demethylated/depropargylated derivatives, respectively.

To find the hydroxylated metabolites of deprenyl, trimethylsilylated derivatives were prepared. This changed the mass fragment belonging to the phenolic ring which contained not only the cycloheptatrienium ion but also its trimethylsilylhydroxy derivative, having a molecular ion of m/z 179. These studies proved that a considerable amount of hydroxymethamphetamine and trace amounts of hydroxypropargylanara were produced during the metabolism of deprenyl, in addition to the earlier known metabolites such as propargylanara, methamphetamine and amphetamine (Fig. 7).

To find the position of (phenolic) hydroxylation during metabolism, some *ortho-*, *meta-* and *para*-methylated deprenyl analogues were investigated. After the administration of the drugs, collection of urine and prepurification by extraction, acetyl derivatives were prepared. With hydroxylated metabolites of the *ortho-* and *meta-*

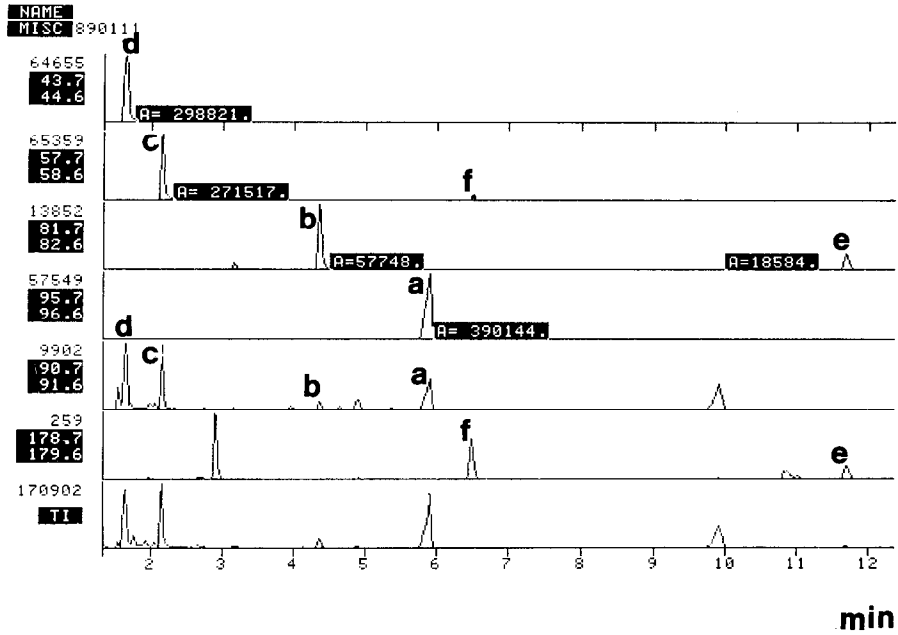

Fig. 7. GC–MS identification of deprenyl metabolites after trimethylsylation. Peaks a, b, c, d, e and f are deprenyl, propargylanara, methamphetamine, amphetamine, *p*-hydroxypropargylanara and *p*-hydroxymethamphetamine, respectively. For the white numbers in the black boxes, see legend to Fig. 5. Numbers equal to A are the relative amounts of ion fragments.

substituted deprenyl derivatives, the presence of hydroxylated metabolites was detected, whereas they were absent when the metabolites of the *para*-substituted drug were analysed (Fig. 8).

DISCUSSION

Serum binding of (−)-deprenyl is probably a strong interaction, as the binding remained unaffected by several hours of gel chromatography. The blood level of (−)-deprenyl shows a remarkably fast onset and decline. The maximum deprenyl level in the brain of mice was observable 30 after intravenous injection, whereas the brain and blood levels of (−)-deprenyl decreased to several percent of the peak level after 2 h of intravenous administration to mice.

After 3 weeks of pretreatment with deprenyl, rats showed a highly enhanced dopamine release in the brain striatal slices. This may be important in the explanation of the mechanism of action of (−)-deprenyl.

Ehringer and Hornykiewicz[15] observed that in the brain of Parkinsonian patients the striatal dopamine concentration is lower than that in normal brain. This is probably due to the loss of dopaminergic neurons, and this loss is compensated by glial cells yielding the monoamine oxidase enzyme. Dopaminergic modulation in the brain and especially in the striatum and hypothalamic area declines in senescence. Moreover, an increased monoamine oxidase B activity can be found. This age-related biochemical lesion can be corrected by (−)-deprenyl[3,4,13].

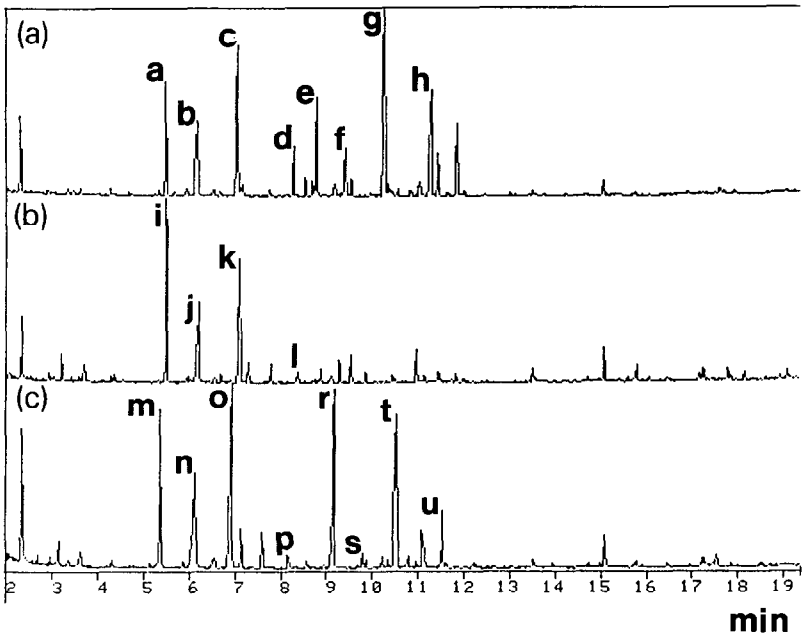

Fig. 8. GC–MS of (A) *o*-methyldeprenyl, (B) *p*-methyldeprenyl and (C) *m*-methyldeprenyl metabolites. Peaks: a = 2-methyldeprenyl; b = 2-methylamphetamine; c = 2-methylmethamphetamine; d = 2-methylpropargylanara; e = 4-hydroxy-2-methyldeprenyl; f = 4-hydroxy-2-methylamphetamine; g = 4-hydroxy-2-methylmethamphetamine; h = 4-hydroxy-2-methylpropargylanara; i = 4-methyldeprenyl; j = 4-methylamphetamine; k = 4-methylmethamphetamine; l = 4-methylpropargylanara; m = 3-methyldeprenyl; n = 3-methylamphetamine; o = 3-methylmethamphetamine; p = 3-methylpropargylanara; r = 4-hydroxy-3-methyldeprenyl; s = 4-hydroxy-3-methylamphetamine; t = 4-hydroxy-3-methylmethamphetamine; u = 4-hydroxy-3-methylpropargylanara.

Reynolds *et al.*[16] also investigated the deprenyl metabolism by GC. They identified amphetamine and methamphetamine in the urine of patients but neither demethylated deprenyl (propargylanara) nor hydroxylated derivatives of deprenyl were found.

The metabolism of deprenyl was explored by displacement TLC. The results indicate the presence of some fragments that are the products of oxidative demethylation, depropargylation and demethylation/depropargylation. However, some additional spots were also present that can be considered to be products of *para*-hydroxylation of either deprenyl or its identified metabolites.

Verification of the presence of dealkylated deprenyl metabolites was obtained by GC–MS. Amphetamine, methamphetamine and propargylanara were found to be metabolites of deprenyl (Fig. 3). Similar experiments were carried out to identify the metabolites of J-508 and TZ-650 (Figs. 4 and 5). The GC–MS identification of further metabolites required derivatization. Acetylation of the urine extract produced adequately volatile derivatives. Their GC–MS spectra showed the presence of *p*-hydroxypropargylanara and a trace amount of *p*-hydroxymethamphetamine among the metabolites (Fig. 7). Similar findings have been published by Yoshida *et al.*[17].

Experiments with *para*-substituted deprenyl derivatives definitely proved that

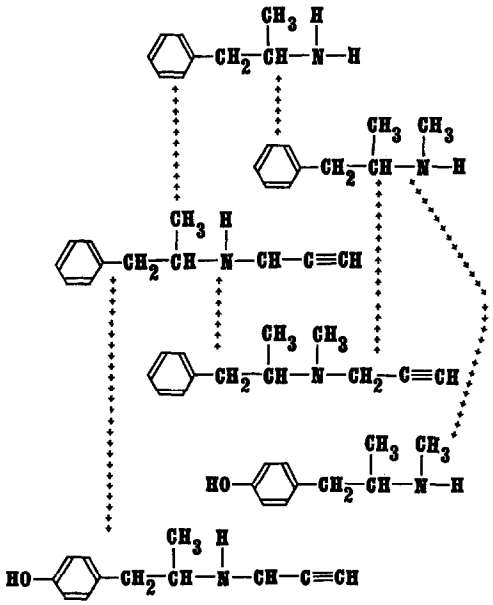

Fig. 9. Metabolic pathway of deprenyl. a, b, c, d, e and f are deprenyl, propargylanara methamphetamine, amphetamine, *p*-hydroxypropargylanara and *p*-hydroxymethamphetamine, respectively.

hydroxylation takes place at the *para* position of the deprenyl, if this process is not hindered by the presence of a *para* substituent in the parent drug (Fig. 8). The metabolic pathway of (−)-deprenyl is shown in Fig. 9.

It is worth mentioning that the very fast onset and decline of the deprenyl level in the brain strongly suggest that the parent drug, (−)-deprenyl, is responsible for the therapeutic effect and that the metabolites do not play any role.

ACKNOWLEDGEMENT

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