Determination of the Norepinephrine Level by High-Performance Liquid Chromatography to Assess the Protective Effect of MAO-B Inhibitors Against DSP-4 Toxicity

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Abstract

Liquid chromatography (LC) combined with electrochemical detection (EC) is suitable for measuring oxidizable biogenic amine levels in small samples of brain tissue. The norepinephrine (NE) content in mouse hippocampus after treatment with various monoamine oxidase-B enzyme (MAO-B) inhibitors ([–]-deprenyl, [+]-rasagiline, and the noradrenergic neurotoxin *N*-[2-chloroethyl]-*N*-ethyl-2-bromobenzylamine [DSP-4]) is determined using an LC–EC method. Treatment with a single intraperitoneal dose of (–)-deprenyl (selegiline) before DSP-4 administration markedly reduces the NE depleting effect of the toxin, and (+)-rasagiline does not significantly modify the NE level decreased by the neurotoxin. The MAO-B inhibitory potency of (–)-deprenyl and (+)-rasagiline is also evaluated. Significantly reduced MAO-B enzyme activity in mouse brain and liver is measured 6 h after treatment with their single dose. (+)-Rasagiline is found to be a more potent MAO-B inhibitor than (–)-deprenyl.

Introduction

Although the presence of norepinephrine (NE) in brain tissue had been demonstrated biochemically in the 1950s and its transmitter role suspected, detailed analysis of its neuronal distribution only became possible when the fluorescent technique (based on the formation of a fluorescent derivative of catecholamines when tissue is exposed to formaldehyde) was devised by Falck and Hillarp (which is based on the formation of a fluorescent derivative of catecholamines when tissue is exposed to formaldehyde). Control noradrenergic systems originate from the pontine tegmentum, the locus ceruleus, and locus subceruleus (1,2). Numerous analytical methods have been elaborated to measure the NE level in different parts of the brain. The NE level can be measured by gas chromatography–electron capture detection (EC) (3,4), gas chromatography–mass spectrometry (5,6), high-performance liquid chromatography (LC)–ultraviolet detection (7), LC–fluorometric detection (8), LC–electrochemical detection (9,10), and radioenzymatic assay methods (11,12). The LC–EC method is popular because of its simplicity. It has been suggested that the selectivity and sensitivity of this method can be further enhanced by the modification of the detector assembly, thus LC–EC became the method of choice (10). EC is intrinsically highly sensitive and selective compared with other analytical techniques, and the analysis of biological samples containing a considerable amount of various contaminants is usually possible with minimum sample pretreatment. In this study an LC–EC method was used to determine the neurotoxic effect of the selective noradrenergic toxin *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4), which causes severe and long-lasting depletion of NE content in various parts of a rodent brain (13,14). We also investigated the possibilities to eliminate DSP-4 toxicity.

Experimental

Chemicals

(–)-Deprenyl (*N*-methyl-*N*-propinyl-[2-phenyl-1-methyl]-ethylammonium chloride) was obtained from Chinoin Pharmaceutical Works (Budapest, Hungary). (+)-Rasagiline (*N*-propinyl-1-amino-indane) and DSP-4 were kindly donated by TEVA (Debrecen, Hungary) and Svante B. Ross (Södertälje, Sweden), respectively. β-Phenylethylamine•HCl (β-PEA) and the chemicals for the HPLC–EC assay (perchloric acid, sodium-bisulfite, 3,4-dihydroxybenzylamine [DHBA], methanol, disodium-phosphate, citric acid, ethylenediaminetetraacetate [EDTA], and octane-sulfonic acid) were obtained from Sigma (Budapest, Hungary). 2-Phenyl-(1-^{14}C)-ethylamine•HCl (^{14}C-

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PEA) (specific activity of 41.8 mCi/mmol) was purchased from Du Pont de Nemours GmbH (NEN Life Science Products, Boston, MA).

Animals

Male NMRI mice (20–25 g) (TOXICOOP, Budapest, Hungary) were housed five in a cage in the animal house under a 12:12 light–dark cycle at 22°C ± 1°C. Tap water and standard mice pellets were available ad libitum. Experiments were started after an adaptation period of at least 2 days.

Treatment and tissue preparation for the measurement of the hippocampal NE level

DSP-4 was applied as a single 50-mg/kg intraperitoneal (i.p.) injection. (–)-Deprenyl and (+)-rasagiline (1 mg/kg, i.p.) were administered 1 h before DSP-4. In all cases mice were decapitated after 7 days from the treatment with the toxin. The substances were dissolved in distilled water immediately before administration. Control animals received the same volume of vehicle. After the mice were sacrificed, the brain was removed and the hippocampal region was dissected on an ice-chilled aluminum surface and stored at –80°C. The tissue samples were sonicated for 30 s in 100 µL of cold 0.2M perchloric acid containing 0.1% $Na_2S_2O_3$ and 3 ng/20 µL DHBA as the internal standard. After centrifugation for 20,000 rpm at +4°C for 20 min, the supernatant (20 µL) was removed and the concentration of NE in the supernatant was determined by the LC–EC method.

Apparatus

The LC apparatus consisted of a pump (PU-1580) (Able & Jasco, Budapest, Hungary), a Rheodyne injector (Able & Jasco) with a 20-µL injector sample loop, a C_{18}-filled guard column (Hypersil ODS 5, 4 × 4 mm) (Sigma-Aldrich Kft., Budapest, Hungary), and a SupelcoSil reverse-phase column (LC-18-DB, 3 µm, 25 x 4.6 mm) (Supelco, Sigma-Aldrich Kft.). A Decade amperometric detector (Able & Jasco) with a glassy carbon electrode (VT-03 analytical cell) was used for EC detection.

Chromatographic conditions

The mobile phase (isocratic elution) contained 5% methanol (v/v) in a 0.05M phosphate–citrate buffer of pH 3.2, which contained 0.025mM EDTA and 1mM octanesulfonic acid. The flow rate of the mobile phase was 1.0 mL/min and the injection volume was 20 µL. External standards were regularly injected in order to identify and calibrate the peaks from the injected samples. The standards were diluted from a stock solution (1 mg/mL) with 0.2M perchloric acid containing 0.1% $Na_2S_2O_3$. The quantitative determination of each standard showed linear regression at the concentration range from 0.1 ng/20 µL to 10 ng/20 µL, and the equation of the calibration line was $y = -0.0215 + 4.2573x$ and the correlation coefficient (R) = 0.9999. The limit of detection was 0.1 ng/20 µL.

Treatment schedule to measure monoamine oxidase-B enzyme activity

Mice were treated i.p. with single doses of 1 mg/kg (–)-deprenyl and (+)-rasagiline. Before treatment, animals were deprived of food for 16 h, but they had free access to water. The animals were sacrificed by decapitation 6 h after drug administration. The liver and brain were dissected on an ice-chilled aluminum surface. The nuclei-free homogenate of the organs was used for the determination of enzyme activity.

Determination of monoamine oxidase-B enzyme activity

Monoamine oxidase–B enzyme (MAO-B) activity was determined radiometrically based on the method of Wurtman and Axelrod (15) with slight modification (16). ^{14}C-PEA was used as a substrate. Protein concentration was measured according to the method of Lowry et al. (19).

Statistics

In order to keep the resulting experimental error at a constant level, data were analyzed by one-way analysis of variance (Statistica 5.0 for Windows, Stat Soft Inc., Tulsa, OK) followed by post hoc analysis. Each treatment group was compared with each other by the method of least significant difference, and the conventional level of $p < 0.05$ was used as the criterion of significance.

Results

The NE content in mouse hippocampus was determined by an LC–EC method. The chromatogram of the NE standard is shown in Figure 1. A single i.p. injection of DSP-4 (50 mg/kg) induced an 80–90% reduction of hippocampal NE content in mice 1 week after treatment. An i.p. administration of 1 mg/kg (–)-deprenyl 1 h before DSP-4 injection considerably reduced the NE-depleting effect of the toxin. Pretreatment with the other MAO-B inhibitor (1 mg/kg [+]-rasagiline) did not affect the DSP-4-induced NE depletion (representative chromatograms after various treatments are shown in Figure 2). The mean NE contents measured in the hippocampus after various treatment schedules are summarized in Table I.

The inhibition of MAO-B enzyme activity in the brain and liver measured 6 h after the i.p. treatment of mice with either 1 mg/kg (–)-deprenyl or (+)-rasagiline is shown in Figure 3. Both compounds induced more than 80% enzyme inhibition in the brain. In the liver, the MAO-B inhibition produced by (+)-rasagiline was significantly higher.

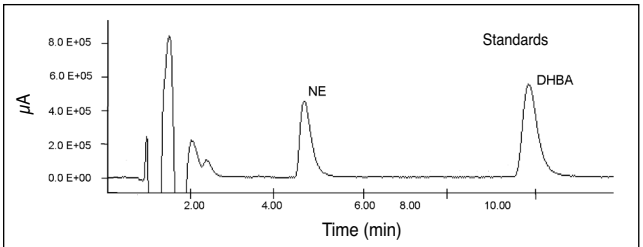

Figure 1. Representative chromatogram of NE standard (1 ng/20 µL) and the internal standard DHBA (1 ng/20 µL). For details see chromatographic conditions.

Discussion

The LC–EC method combination has become the method of choice for the determination of tissue biogenic amines and their metabolites. This technique has the advantages of sensitivity, selectivity, and is more favorably applicable for the determination of low amounts of catecholamines in biological fluids (17,18) and tissue homogenates (19,20). LC–EC has several attractive features when compared with both classical fluorescence and radioenzymatic methods. The technique is rapid and selective. The sensitivity approaches that of the previous methods for most types of samples. LC–EC combines the selectivity of LC with that of electrochemistry, resulting in a highly specific and sensitive assay. This opens the field for the analysis of a wide variety of pharmaceuticals, agricultural chemicals, and industrial intermediates as well. Some of these compounds have physiological or pathological importance, and LC–EC is a useful tool in neurotoxicological and neuropharmacological works.

The neurotoxin DSP-4 causes severe and long-lasting depletion of NE content in various parts of a rodent brain (13,14). This effect is most prominent in brain areas that obtain the majority of their NE terminals from the locus ceruleus (21). One of the targets of DSP-4 action seems to be the carrier system of NE transport into the neurons (22). Previously published data showed that DSP-4 (a tertiary amine) can spontaneously generate a quaternary ammonium derivative in aqueous media, whose positively charged structure was suggested to be responsible for the long-lasting impairment of the NE uptake process, which in turn causes depletion of NE (22). The reduced amount of NE after DSP-4 treatment can be analyzed reproducibly and accurately by LC–EC.

Previous studies showed that the neurotoxic effect of DSP-4 can be inhibited by pretreatment with NE uptake blockers, such as desipramine, cocaine, or zimelidine (13). We intended to study whether any other (not NE uptake blocker) substances are capable of diminishing the effect of DSP-4. (–)-Deprenyl, which is a selective, irreversible inhibitor of the MAO-B (23) and is widely used alone or in combination with L-3,4-dihydroxyphenylalanine in the treatment of Parkinson's disease (24), can provide protection against selective dopaminergic (MPTP, 6-hydroxydopamine), cholinergic (AF64A), and noradrenergic (DSP-4) toxins as well. According to this study, (–)-deprenyl in a single 1-mg/kg i.p. dose 1 h before DSP-4 injection almost completely prevented the NE-depleting effect

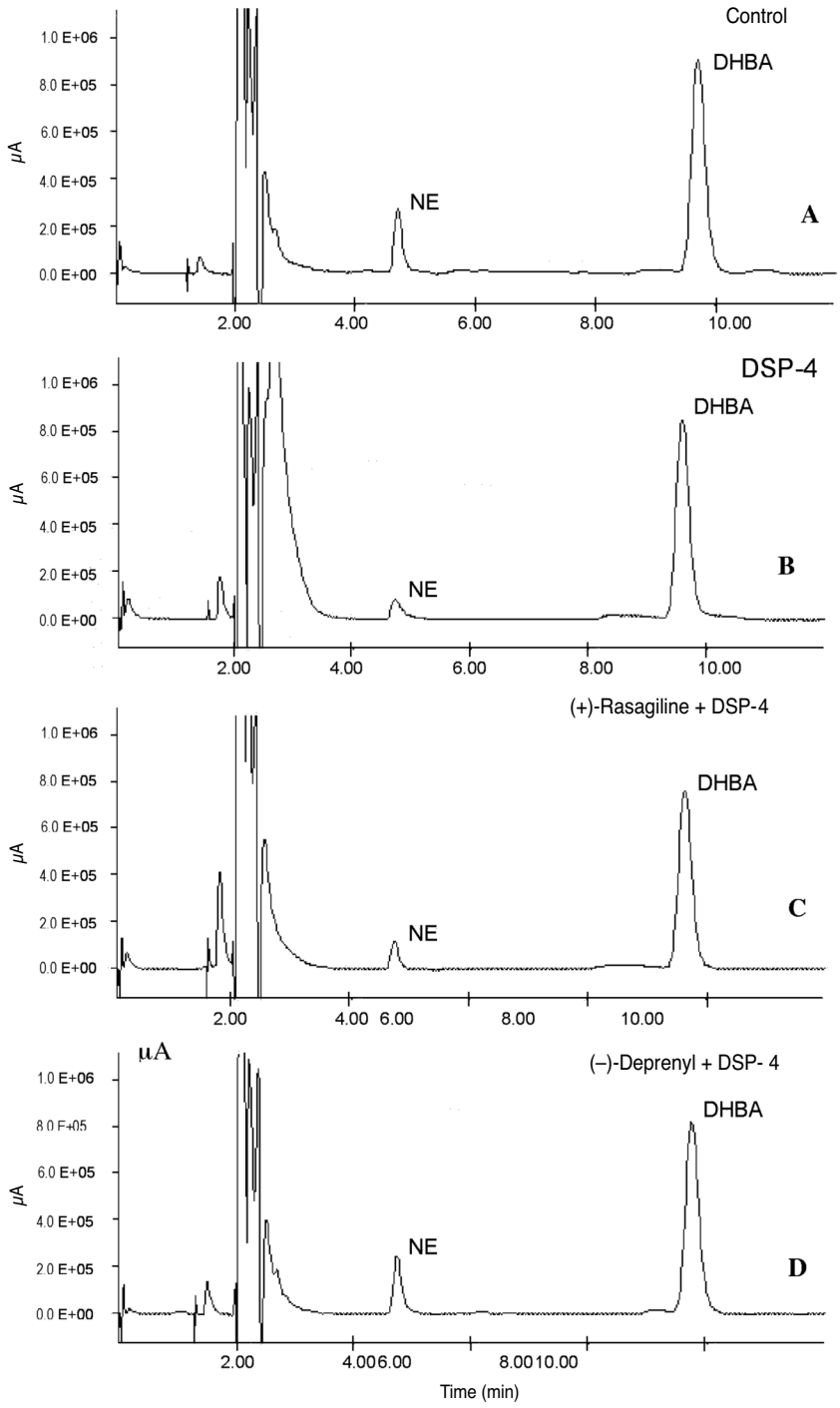

Figure 2. Representative chromatograms of NE extracted from mice hippocampus after various treatment schedule: (A) NE level after treatment with saline; (B) NE level 7 days after DSP-4 (50 mg/kg i.p.) treatment; (C) NE level 7 days after pretreatment with (+)-rasagiline (1 mg/kg i.p.) and treatment with DSP-4 (50 mg/kg i.p.); (D) NE level 7 days after pretreatment with (–)-deprenyl (1 mg/kg i.p.) and treatment with DSP-4 (50 mg/kg i.p., time between pretreatment and treatment was one hour in each case).

of the toxin. The protective effect of (–)-deprenyl against the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine can be explained by the MAO-B inhibitory potency of the substance (25). Supposing that the MAO-B inhibitory action of the compound is related to its protective effect against DSP-4, other enzyme inhibitors should also be protective. (+)-Rasagiline is more potent to inhibit MAO-B enzyme, and its effect on brain MAO-B activity lasts longer than that of (–)-deprenyl. However, (+)-rasagiline pretreatment did not influence the effect of the toxin significantly. Based on these results, the effect of (–)-deprenyl against DSP-4 is unrelated to MAO-B enzyme inhibition. This finding is in agreement with that of Finegan (26) when (–)-deprenyl pretreatment 24 h before DSP-4 was not effective in preventing NE depletion, although MAO-B inhibition was still complete when the toxin was injected.

Several hypotheses have been proposed regarding the neuroprotective mechanism of (–)-deprenyl. One of them has suggested that (–)-deprenyl can inhibit the uptake of biogenic amines (27,28) and such an effect may be relevant in its protection against DSP-4 (12). (–)-Deprenyl is a structural analog of (–)-methylamphetamine ([–]-MA) and is converted to (–)-MA, (–)-desmethyldeprenyl, and (–)-amphetamine ([–]-A) by CYP-450 enzymes in the liver (29–31). The main metabolites of (–)-deprenyl, (–)-MA, and (–)-A are more effective uptake blockers than the parent compound (32), but they can also enhance the release of NE and dopamine from the nerve endings (33). The possible contribution of the metabolites to the protective effect of (–)-deprenyl against DSP-4-induced NE depletion has also been suggested (34). The mechanism of the protective effect of (–)-deprenyl needs to be further clarified.

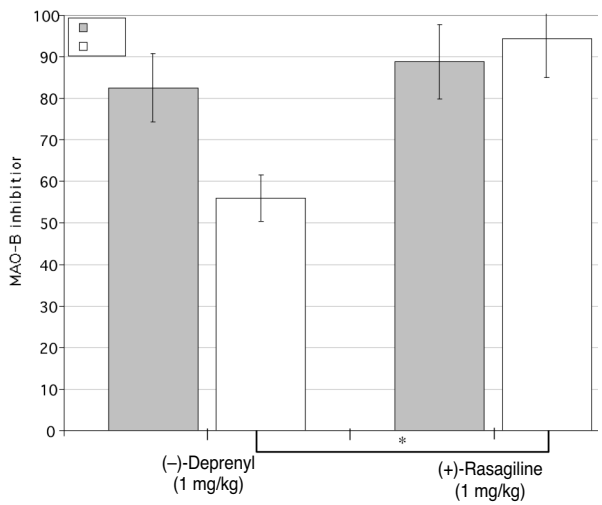

Figure 3. The effect of (–)-deprenyl and (+)-rasagiline (1 mg/kg i.p.) on MAO-B activity in nuclei free homogenate of mouse brain and liver (n = 3) 6 h after the treatment (mean ± standard deviation). There is a significant difference between the columns marked with an asterisk ($p < 0.05$).

Table I. The Effect of (–)-Deprenyl and (+)-Rasagiline on DSP-4-Induced NE Depletion in Mouse Hippocampus*

Treatment (n = 5)	NE content (ng/mg tissue) (mean ± SD†)	NE content in % of control (mean)
Control	0.88 ± 0.06	100
DSP-4 (50 mg/kg)	0.08 ± 0.01	9.1
(+)-Rasagiline (1 mg/kg i.p. + DSP-4 (50 mg/kg)	0.102 ± 0.2	11.6
(–)-Deprenyl (1 mg/kg i.p.) + DSP-4 (50 mg/kg)	0.73 ± 0.08	83

* There is no significant difference between the control and the (–)-deprenyl pretreated groups ($p > 0.05$).
† SD, standard deviation.

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