HPLC and HPLC–MS Analysis of Urinary N^{ε}-Monomethyl-Lysine

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Abstract

Administration of ^{14}C-labelled L-deprenyl to rats results in the urinary elimination of a ^{14}C-labelled compound. The 9-fluorenylmethoxycarbonyl chloride-reacted urine sample is fractionated by high-performance liquid chromatography (HPLC) on an octadecyl silica stationary phase. N^{ε}-Monomethyl-lysine is identified in the fraction containing the majority of the radioactivity. Structural elucidation is carried out using HPLC–mass spectrometry in atmospheric pressure chemical ionization mode. Identification of the ^{14}C-labelled fragment in N^{ε}-monomethyl-lysine is an experimental proof that an N-methylated amino acid is generated by transmethylation from a well-known drug. This type of transmethylation may have basic importance in the positive side effects of certain drugs.

Introduction

L-Deprenyl (selegiline hydrochloride) is an irreversible, potent, and specific inhibitor of monoamine oxidase B enzyme. It is used in the treatment of symptoms of Parkinson's disease (1) and is commercially available under various registered names such as Jumex, Movergan, Eldepryl, etc.

The metabolic pathways of deprenyl have been investigated on a wide variety of animal species, in addition to human subjects. The metabolism of L-deprenyl starts with its N-oxidation, which yields deprenyl *N*-oxide (2). Further metabolic processes of L-deprenyl and its metabolites include hydroxylation and N-depropynylation to L-methamphetamine. L-Methamphetamine is subjected to demethylation, yielding the norcompound (L-amphetamine) and also formaldehyde. The main metabolic pathways of deprenyl are given in Figure 1. Formaldehyde is urinary eliminated (3–5) or can take place in further reactions.

A reliable method for metabolic study is to prepare the radiolabelled parent drug and to trace radioactivity. The radiolabelled metabolites can be detected on a thin-layer chromatographic (TLC) plate using digital autoradiography, an x-ray film, or other contact detection methods. Any metabolites having the radiolabelled part of the compound can be localized, even the compound remaining at the start, or migrating together with the solvent front (6,7). The presence of radioactivity in the spot is a sure indicator that it originated from the radiolabelled parent drug. The chemical structure of the compounds forming the spots can generally be elucidated by the use of hyphenated methods, such as TLC–mass spectrometry (MS) or high-performance liquid chromatography (HPLC)–MS.

When rats were treated with the radiolabelled L-deprenyl, a high portion of radioactivity was eliminated in the urine. The well-known metabolites of L-deprenyl were identified in the urine using TLC and HPLC–MS; however, certain unidentified spots were also found around the start on the TLC plate.

The hitherto published metabolic studies of L-deprenyl concerned metabolites whose chemical structure contained an essential part of the parent compound. This paper deals with the methods resulting in the identification of N^{ε}-monomethyl-L-lysine as one of the metabolites. Only a minor part of its structure (the methyl group) originated from L-deprenyl.

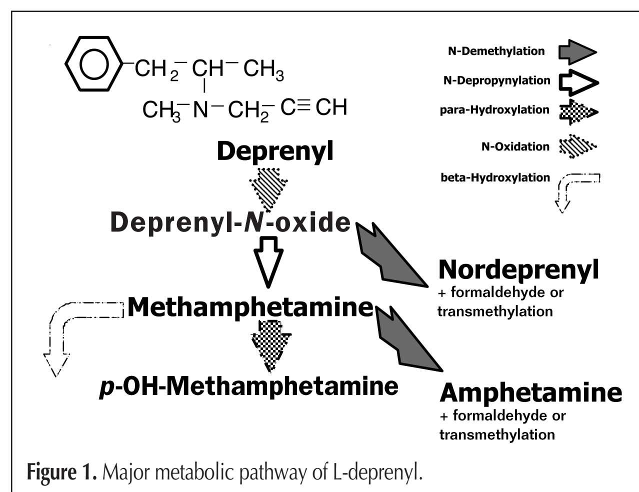

Figure 1. Major metabolic pathway of L-deprenyl.

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Experimental

Materials

Solvents and chemicals

Solvents of HPLC-grade purity were purchased from E. Merck (Darmstadt, Germany). HPLC-grade water was used. L-Deprenyl [selegiline hydrochloride; (–)-*N*-methyl-*N*-propynyl(2-phenyl-1-methyl)ethylammonium hydrochloride, also called (R)-(–)-*N*,2-dimethyl-*N*-2-propynylphenethylamine hydrochloride] was the kind gift of Chinoin Pharmaceutical and Chemical Works (Budapest, Hungary; presently, a member of the Sanofi-Sintelabo Group). ^{14}C-L-deprenyl [(–)-^{14}C-*N*-methyl-*N*-propynyl(2-phenyl-1-methyl)ethylammonium hydrochloride, 98 µCi/mg] was prepared from (–)-*N*-propynyl(2-phenyl-1-methyl)ethylammonium hydrochloride and supplied by the Institute of Isotopes Co., Ltd. (Budapest, Hungary).

N^{ε}-Methyl-L-lysine HCl, (Serva Feinbiochemica, Heidelberg, Germany) and 9-fluorenylmethoxycarbonyl chloride (Fmoc chloride) (Sigma, St. Louis, MO) were used. Insta-Fluor cocktail was purchased from Packard Instruments (Groeningen, the Netherlands). All chemicals were of the highest purity grade available.

Instrumentation

HPLC

A JASCO system (JASCO, Tokyo, Japan) was used to separate the urine samples after derivatization with Fmoc hydrochloride. It contained a DG-208054 degasser, two PU-1580 pumps, an AS-2057 plus automatic sample injector, and a UV-1575 detector. Data were stored and analyzed with an SRI Model 202 peak simple chromatographic data system (SRI Instruments, Torrance, CA). The HPLC separation was carried out using a stainless steel column (25-cm × 4.6-mm i.d.) packed with 6-µm Kovasil C18 endcapped particles (Chemie Uetikon, Uetikon, Switzerland). Acetonitrile–water (2:1) also containing 0.1% formic acid was used as the mobile phase. The column was stored at 28°C, the mobile phase flow rate was 1.5 mL/min, and each fraction was collected for 1 min.

HPLC–MS

A model HP LC–MS 1100 instrument (Agilent, Waldbronn, Germany) was used for the measurements. It was equipped with a high-pressure gradient pump and diode-array detector, and the MS was used in the positive atmospheric pressure chemical ionization mode. Injection volumes were 1 µL of the monomethyl lysine standard sample and 25 µL of the urine samples. The vaporizer temperature, drying gas flow rate, and nebulizer pressure were 350°C, 5 L/min, and 40 psig, respectively.

The HPLC separation was carried out using a stainless steel column (120 × 4 mm) packed with 5 µm Eurospher-100 C18 endcapped particles (Knauer, Berlin, Germany). The column was thermostated at 40°C. Acetonitrile–water (6:1) also containing 0.1% formic acid was used as the mobile phase.

Methods

Male Wistar rats (200–250 g) were treated per os with radiolabelled L-deprenyl. Urine samples were collected for 6 h.

Precolumn derivatization with Fmoc chloride

To each 1-mL urine sample, 0.5 mL potassium borate buffer (0.8M, pH 10) was added, and the mixture was vigorously shaken for 1 min using a mixer. One milliliter of 10mM Fmoc chloride solution in acetonitrile was then added and immediately vortexed for 1 min. One milliliter of *n*-hexane was then added, the mixture was shaken for 1 min, and the upper and lower phases were separated by centrifugation. The upper (organic) phase containing the excess Fmoc reagent was discarded. The *n*-hexane extraction of the excess Fmoc was repeated twice. Finally, 100 µL of acetic acid (10%, v/v) was added to the samples, mixed, and 100 µL of sample was subjected to HPLC separation. A similar derivatization was performed with the standard compounds.

Determination of radioactivity

Five milliliters of Insta-Fluor cocktail was given to 100 µL of the 1.5-mL HPLC fraction.

Results

A 1:1 mixture of acetonitrile–water also containing formic acid is an adequate mobile phase when using octadecyl silica stationary phase. Detection at 265 nm gives certain specificity.

Calibration shows a linear dependence of peak area versus load in the range of 0.1 mg/mL through 0.1 µg/mL of N^{ε}-methyl-L-lysine HCl. This linearity means reliable detection from 2.5 µg through 2.5 ng of the compound in a 25-µL sample.

Urine was collected after the administration of ^{14}C-labelled L-deprenyl to the rats. The sample was derivatized with Fmoc chloride and subjected to reversed-phase chromatographic separation. The fractions were collected for 1 min each, and their radioactivity was determined. Figure 2 plots fraction numbers versus radioactivity values. There is a major peak of radioactivity in fraction no. 16. This fraction was reanalyzed by HPLC with MS detection. Figure 3 shows the chromatograms detected at 265 nm (top chromatogram), total ion current (TIC, center chromatogram), and selected ion monitoring (SIM, at 383 amu, bottom chromatogram). HPLC–MS of a standard Fmoc-N^{ε}-monomethyl-lysine sample was also recorded (Figure 4). Data

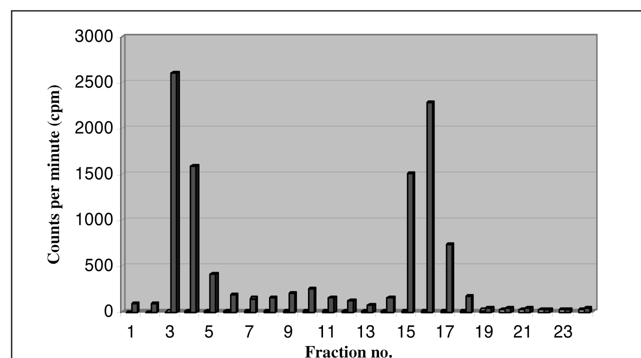

Figure 2. HPLC of rat urine sample reacted with Fmoc chloride. The separation was performed using 6-µm Kovasil C18 endcapped particles (25-cm × 4.6-mm i.d) and acetonitrile–water (2:1, also containing 0.1% formic acid) as the mobile phase. The mobile phase flow rate was 1.5 mL/min. Fraction number versus radioactivity (counts per minute) values. Fraction no. 16 was collected from 15 to 16 min.

show the presence of N^{ε}-monomethyl-lysine in the fraction containing the majority of radioactivity. The mass spectrum of standard N^{ε}-monomethyl-lysine and that of the radioactive fraction are shown in Figure 5. The UV spectrum of the standard Fmoc-N^{ε}-monomethyl-lysine and that of fraction no. 16 were also recorded. These spectra were practically the same (Figure 6).

Discussion

Metabolites are the compounds resulting from a xenobiotic substance after it has been subjected to certain alterations of its chemical structure in the body. Such biochemical reactions are called metabolism, and the gross routes of metabolism are specified as metabolic pathways. These chemical alterations can be theoretically outlined even using mathematical models with the help of computers (8). However, the classical and generally accepted method of establishing the metabolic pathways is chromatography. Advantageous effects of L-deprenyl in neuroprotection have been claimed to the deprenyl metabolites (9). An exceptionally low concentration of L-deprenyl has an influence on cell proliferation. Magyar et al. (10) detected dose-dependent stimulation/inhibition on the apoptosis, and even 10^{-12} mol concentration of L-deprenyl was effective. This phenomenon cannot be explained by direct action, however, peculiar metabolites may be responsible.

Numerous reports have dealt with the metabolism of L-deprenyl [for review, see (11)]. Gas chromatography (GC) was widely employed to separate metabolites, and identification was carried out by either comparison with the standards of tentative metabolites or using GC–MS. Nor-deprenyl, amphetamine, methamphetamine, *p*-hydroxy-methamphetamine, phenylacetone, and several other deprenyl metabolites have been detected in this way. GC, however, generally requires derivatization of the metabolites (7).

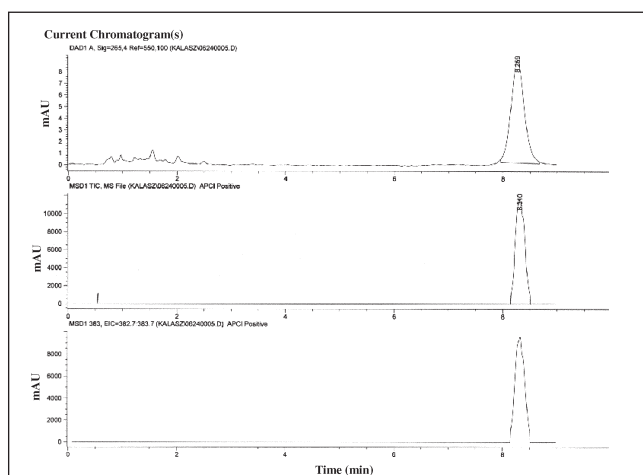

Figure 3. Three different types of monitoring of the chromatography of fraction no. 16. (from a load of 25 µL). Detections were carried out at 265 nm (top chromatogram), by TIC (center chromatogram), and by SIM (at 383 amu, bottom chromatogram).

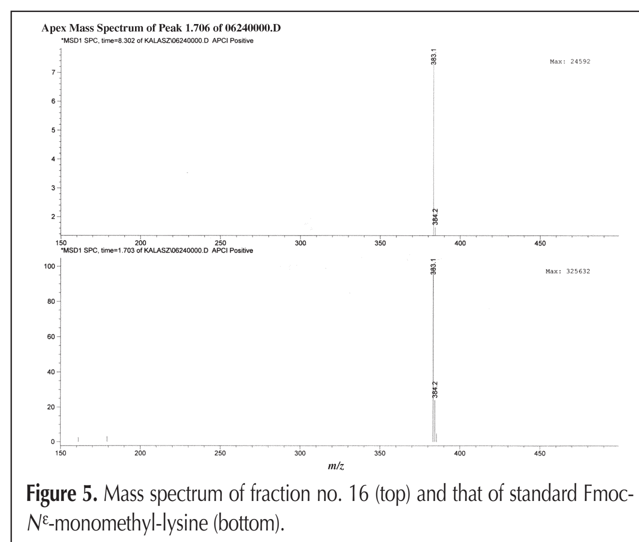

Figure 5. Mass spectrum of fraction no. 16 (top) and that of standard Fmoc-N^{ε}-monomethyl-lysine (bottom).

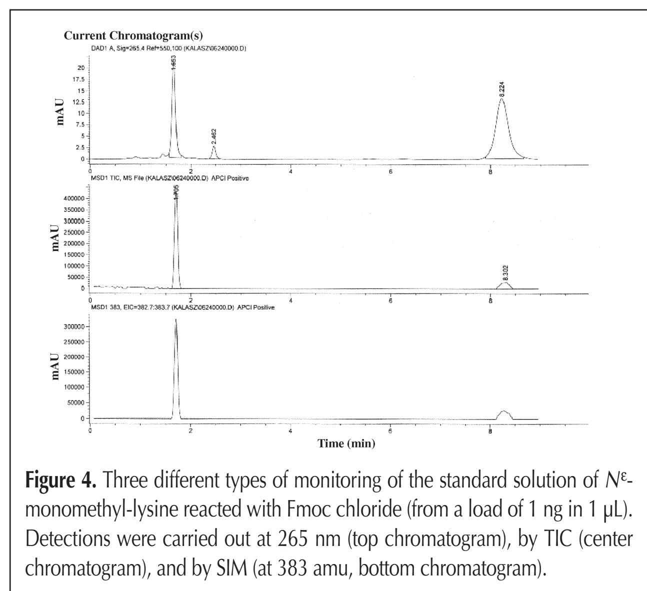

Figure 4. Three different types of monitoring of the standard solution of N^{ε}-monomethyl-lysine reacted with Fmoc chloride (from a load of 1 ng in 1 µL). Detections were carried out at 265 nm (top chromatogram), by TIC (center chromatogram), and by SIM (at 383 amu, bottom chromatogram).

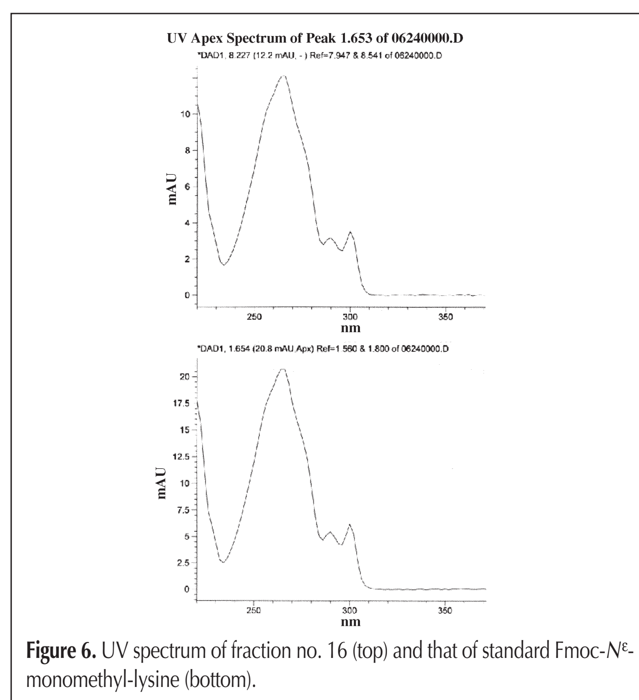

Figure 6. UV spectrum of fraction no. 16 (top) and that of standard Fmoc-N^{ε}-monomethyl-lysine (bottom).

Tábi et al. (12) separated the chiral isomers of tentative and real metabolites of (–)-deprenyl. No change of the chirality was found. If the metabolite is not known, liquid chromatography is the most widely used separation method. Katagi et al. (2) identified deprenyl *N*-oxide as a novel deprenyl metabolite. Their results indicated that deprenyl-*N*-oxide is the major metabolite of urinary elimination.

The problem starts when either a trace level of metabolites has to be determined or earlier unknown metabolites are assumed to be present. Radiolabeling of the parent drug helps to find the metabolites, and HPLC–MS can be employed to establish the chemical structure of the metabolite present in a radioactive fraction.

Kalász et al. (11,13) did not prepare derivatives; they used TLC to characterize the lipophilicity of deprenyl and that of the metabolites. In the same papers (11,13) HPLC was also used to separate the deprenyl metabolites that were identified using electrospray MS. The fate of the *N*-methyl group of L-deprenyl can be specified by three different ways. A major route is urinary elimination of the parent L-deprenyl, as well as of the L-deprenyl *N*-oxide, L-methamphetamine, and also formaldehyde and N^{ε}-monomethyllysine. In another possibility, N-demethylation resulted in the transmethylation to either an amino acid (lysine, arginine, or histidine) or to a DNA or to DNA fragments. The third alternative route can be postulated as the formaldehyde formed during metabolism is further oxidized through formic acid to carbon dioxide, and the carbon dioxide is eliminated by the lung with the exhausted air. The oxidation is catalyzed by the catalase enzyme, and a parallel reaction is decomposition of hydrogen peroxide. N-Demethylation metabolism may therefore be a beneficial way to counterbalance the oxidative stress.

Metabolic transfer of a methyl group is a general phenomenon of living organisms. This process serves the homeostasis, the usual equilibrium of biochemical processes in the healthy living organisms (14). Methylated amino acids, peptides, and proteins have their well-defined physiological functions. The presence of N^{ε}-monomethyl-lysine was earlier found in tissues showing slow proliferation (bradyotropic), such as the tooth (15).

Methylation of DNA has recently come into the limelight. A literature search indicated over 20,000 papers dealing with N-methylation, and the overwhelming majority of these publications were concerned with DNA-methylation. An adequate supply of the methyl group depends on the amount and also the duration of the methyl group in the body, and especially in the site of transmethylation. It can be a reason for the advantageous side effect of L-deprenyl, which stays in the brain for a surprisingly long amount of time.

Conclusion

Experimental results support the formation of N^{ε}-monomethyl-lysine based on the *N*-methyl group of L-deprenyl. The process of methyl transfer was traced with the help of the ^{14}C-methyl group of L-deprenyl, and the detection was facilitated by forming the Fmoc derivative. Preparative separation was carried out using HPLC, but the final structural elucidation was successful with the help of HPLC–MS.

Acknowledgments

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