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Detection of N^{ε}-monomethyllysine using high-performance liquid chromatography and high-performance liquid chromatography–mass spectrometry

H. Kalász [a,*], Z. Szücs [b], M. Tihanyi [a], Á. Szilágyi [a], J. Lengyel [c]

[a] *Department of Pharmacology and Pharmacotherapy, Semmelweis University, Nagyvárad tér 4, H-1089 Budapest, Hungary*
[b] *Research Institute for Medicinal Plants, P.O. Box 11, H-2011 Budakalász, Hungary*
[c] *Central Isotope Laboratory, Semmelweis University, Nagyvárad tér 4, H-1089 Budapest, Hungary*

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Abstract

N^{ε}-Monomethyllysine was identified in the serum, urine, brain, and liver samples of rats treated per os with L-deprenyl. The identification procedure included reaction with Fmoc chloride, clean-up, and analysis using HPLC–UV–MS. Oral administration of (−)-*N*-^{14}C-methyl-*N*-propynyl(2-phenyl-1-methyl)ethylammonium hydrochloride L-deprenyl) to rats resulted in transfer of the radiolabelled methyl group to the N^{ε}-amino group of the endogenous lysine. The radiolabelled N^{ε}-monomethyllysine was urinary eliminated together with the other radiolabelled deprenyl metabolites, such as deprenyl-*N*-oxide and methamphetamine. The presence of N^{ε}-monomethyllysine has also been traced, and its concentrations were compared in the serum, liver and brain of rats subjected to L-deprenyl treatment. Methyl group transfer from the L-deprenyl to endogenous compounds; and the urinary elimination of their products may offer a vital way to eliminate or to decrease the degree of drug transmethylation to the lysine constituents of blood vessels' proteins.

Keywords: L-Deprenyl; N^{ε}-Monomethyllysine; Transmethylation; HPLC; HPLC/MS

1. Introduction

L-Deprenyl (selegiline hydrochloride) has been widely used in the treatment of Parkinsonian patients [1]. L-Deprenyl containing medicines have various registered names, such as Jumex™, Movergan™, Eldepryl™, etc.

Use of HPLC–MS has given a real impetus to the analysis of deprenyl metabolites. Deprenyl-*N*-oxide [2] was discovered as the abundant metabolite of L-deprenyl, the compound which can be the initiative step of oxidative metabolism. The next alteration steps of deprenyl metabolism include *N*-depropynylation leading to methamphetamine [3–5]. Methamphetamine can be subjected to *N*-demethylation yielding its nor-compound (L-amphetamine) and also formaldehyde. Formaldehyde is urinary eliminated [6–9] or can participate in further reactions. The metabolism of L-deprenyl also produces various other products; over 50 deprenyl metabolites have been identified.

A reliable way to scout new metabolites is to use the radiolabelled parent drug, and trace radioactivity. The radiolabelled metabolites are separated using thin-layer chromatography detected by either digital autoradiography, or on an X-ray film. Any metabolite having the radiolabelled part of the compound can be localized, even the compound remaining at the start, or migrating together with the solvent front [6,9]. The presence of radioactivity in the spot is a sure indicator that it is 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–MS or HPLC–MS.

A high portion of metabolites are eliminated with the urine after oral treatment of rats with L-deprenyl. The overwhelming majority of these metabolites has been well known, as

* Corresponding author. Present address: Department of Pharmacology and Therapeutics, United Arab Emirates University, Al Ain, P.O. Box 17666, United Arab Emirates. Fax: +971 3767 2033.
E-mail address: Huba.kalasz@gmail.com (H. Kalász).

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the identification used the possible widest scale of chromatographic methods, such as gas chromatography (GC) [10], thin-layer chromatography (TLC) [9], high-performance liquid chromatography (HPLC) [8] and capillary zone electrophoresis (CE) [11], also in combination with mass spectrometry (MS) [4,12]. Certain hitherto unidentified spots can also found around the start of the TLC plate, when the metabolites of the radiolabelled deprenyl are subjected to in-depth analysis.

Published studies on drug metabolism are aimed to such compounds whose structural elements contain the major part of the parent drug. This paper deals with metabolites with only a minor structural element: it is the methyl group of which is originated from the parent drug, i.e. from L-deprenyl.

2. Experimental

2.1. Solvents and chemicals

All solvents were of HPLC grade purity purchased from E. Merck (Darmstadt, Germany). L-Deprenyl [(*R*)-(−)-*N*,2-dimethyl-*N*-2-propynylphenethylamine hydrochloride] was the gift of Chinoin Pharmaceutical and Chemical Works (Budapest, Hungary; a member of the Sanofi-Sintelabo Group). (−)-[^{14}C]-*N*-Methyl-*N*-propynyl(2-phenyl-1-methyl)ethylammonium hydrochloride [^{14}C-L-deprenyl, 98 μCi mg^{-1}] has been prepared using (−)-*N*-propynyl(2-phenyl-1-methyl)ethylammonium hydrochloride and kindly supplied by the Institute of Isotopes (Budapest, Hungary).

N^{ε}-Monomethyl-L-lysine HCl, p.a. (analytical-reagent grade; MML, Serva, Heidelberg, Germany), Fmoc chloride (9-fluorenylmethoxycarbonyl chloride, Sigma, St. Louis, MO, USA) and Insta-Fluor™ cocktail (Packard Instruments, Groningen, The Netherlands) were used. All other chemicals were of the available highest purity grade.

2.2. Methods

Male Wistar rats (200–250 g) were subjected to oral treatment with radiolabelled L-deprenyl. The urine sample was collected for 6 h, when the rats were sacrificed. Blood sample of rats was taken, and the liver and brain were dissected.

2.3. Pre-column derivatization with Fmoc chloride

One gram of the tissue, plasma and urine samples were homogenized with 1 mL potassium borate buffer (0.8 M, pH 10), and the extract was sedimented by centrifugation at 5000 rpm. One milliliter of 10 mM Fmoc chloride solution in acetonitrile was then added to the supernatant, and vortexed immediately for 1 min. Five milliliters of *n*-hexane was then added, the mixture shaken for 1 min, and the upper and lower phases were separated by centrifugation. The organic (upper) phase containing the excess of Fmoc reagent was discarded. The *n*-hexane extraction was repeated twice to remove the excess Fmoc. After adding 100 μL of acetic acid (10%, v/v) to the samples, 100 μL aliquot samples were subjected to HPLC separation. Similar derivatization was carried out with the standard compounds. No hydrolysis was done prior to the Fmoc reaction.

2.4. Determination of radioactivity

Insta-Fluor™ cocktail was used as detailed in our former publication [8].

2.4.1. HPLC

A JASCO (Tokyo, Japan) system served to separate the urine samples after their derivatization with Fmoc hydrochloride. The system contained a DG-208054 degasser, two PU-1580 pumps, and an AS-2057 plus (thermostated) automatic sample injector. The separation was monitored using a UV-1575 detector. Chromatographic data were analyzed with SRI Model 202 Peak Simple Chromatographic Data System (SRI Instruments, Torrance, CA, USA). HPLC separations were carried out using a 25 cm × 4.6 mm I.D. stainless-steel column packed with 6 μm Kovasil C_{18} endcapped particles (Chemie Uetikon, Uetikon, Switzerland). Acetonitrile–water (66:33) also containing 0.1% formic acid was used as the mobile phase with a flow rate of 1.5 mL min^{-1}. Each fraction was collected for 1 min (1.5 mL). The column temperature was 28 °C.

2.4.2. HPLC–MS

HPLC separation for MS was carried out using a 12 cm × 4 mm stainless-steel column packed with 5 μm Eurospher-100 C_{18} endcapped particles (Knauer, Berlin, Germany), the column was kept at 40 °C. The mobile phase was acetonitrile–water (3:2) also containing 0.1% formic acid.

A model HPLC–UV–MS 1100 instrument of Hewlett-Packard (now Agilent, Waldbronn, Germany) was used for the measurements. The system contained a high-pressure gradient pump and a diode-array detector. The mass spectrometer was used in positive atmospheric pressure chemical ionization (APCI) mode. Injection volumes were either 1 μL (of the monomethyl lysine standard sample) or 25 μL (of the other samples). The vaporizer temperature, drying gas flow rate and the nebulizer pressure were 350 °C, 5 L/min and 40 psig, respectively.

3. Results and discussion

A 2:1 mixture of acetonitrile–water also containing formic acid is an adequate mobile phase when using octadecyl silica stationary phase. The Fmoc reacted urine sample was separated on a 25 cm × 4.6 mm I.D. column packed with 6 μm Kovasil C_{18}. The second radioactive peak was subjected to HPLC/MS and gave a peak with the same elution time and mass spectrum as the standard Fmoc-MML.

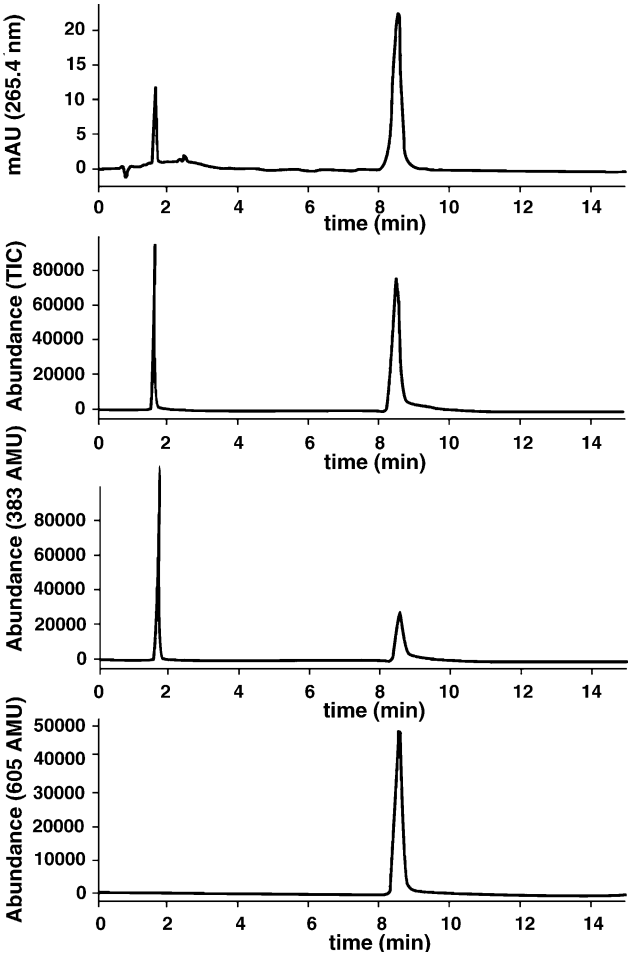

Fig. 1. HPLC–UV–MS detection of standard Fmoc-MML using a 12 cm × 4 mm stainless-steel column packed with 5 μm Eurospher-100 C_{18} endcapped particles, the mobile phase was acetonitrile–water (3:2) also containing 0.1% formic acid. The column effluent was monitored using a diode-array detector at 265.4 nm, total ion current with SIM at 383 u, and SIM at 606 u on the first, second, third and fourth chromatogram (from the top to bottom), respectively.

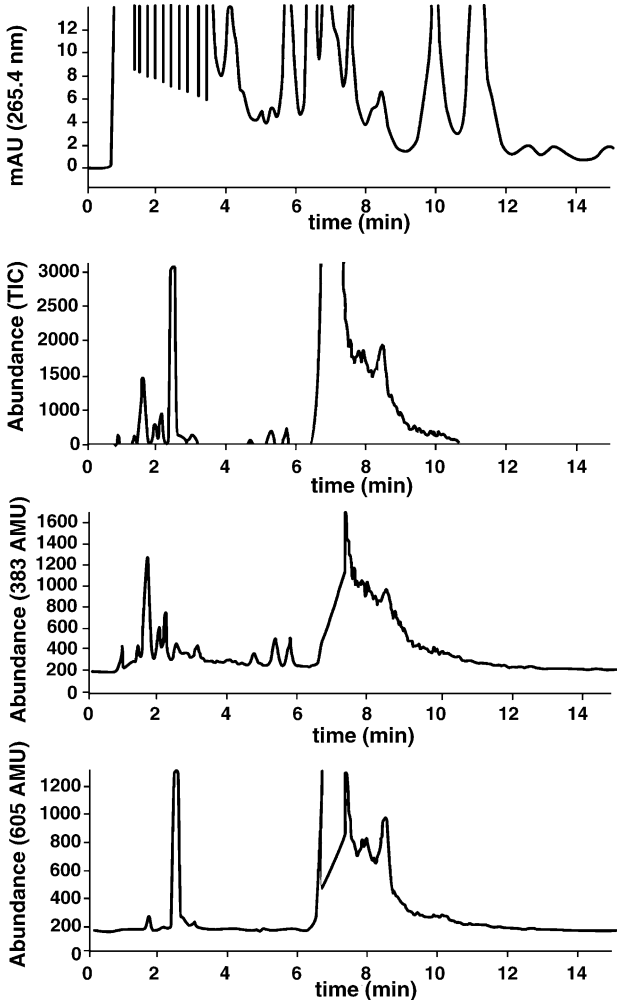

Fig. 2. HPLC–UV–MS detection of Fmoc-MML originated from urine sample of the rat treated with L-deprenyl. Chromatographic conditions and monitoring as detailed for Fig. 1.

HPLC–MS is an excellent tool to scout the MML content of the various samples, such as the urine, serum, liver and brain of the rats treated with L-deprenyl. Fig. 1 gives the elution curves of Fmoc-MML. There were monitored using ultraviolet detection at 265.4 nm, total ion current, with SIM at 383 AMU, and SIM at 605 AMU on the first, second, third and fourth chromatogram (from the top to bottom), respectively. The same detections were used to trace MML in the 1 mL sample of rat urine (Fig. 2), as well as in the 1 g of samples taken from rat plasma (Fig. 3), rat liver (Fig. 4) and rat brain (Fig. 5).

The peak eluted at about 8.5 min is characteristic for these four samples; however, the overlapping peaks are varying from sample to sample and also according to the way of detection. The first estimation indicates a ratio of the MML in the urine–liver–plasma–brain as 31:77:6:297 based on SIM at 383 AMU; however, the ratio is 66:37:3:254 based on SIM at 605 AMU. This discrepancy should be solved by clean-up and further systematic determinations.

The most interesting phenomenon is the peak traffic jam when MML was traced in the urine sample. This was the reason that reliable identification of MML in the urine required its preliminary separation using a 25 cm × 4.6 mm column; also the peak radioactive fraction had to be subjected to the HPLC–UV–MS identification. The results gave direct experimental proofs of the methyl transfer from deprenyl to the epsilon amino group of lysine.

The relatively short period (a few hours) excludes the existence of any multi-step metabolic procedure, such as that the metabolically generated formaldehyde would cross-link two lysine elements of two peptides (or proteins), and the subsequent reactions would generate MML, as observed in the experimental procedure of Yu and co-workers [13]. This reaction requires reduction using sodium borohydride, and this reduction is unlikely to happen in the living organism, at least not in such a short time. Moreover, assuming the release of MML from the cross-linked two peptide molecules,

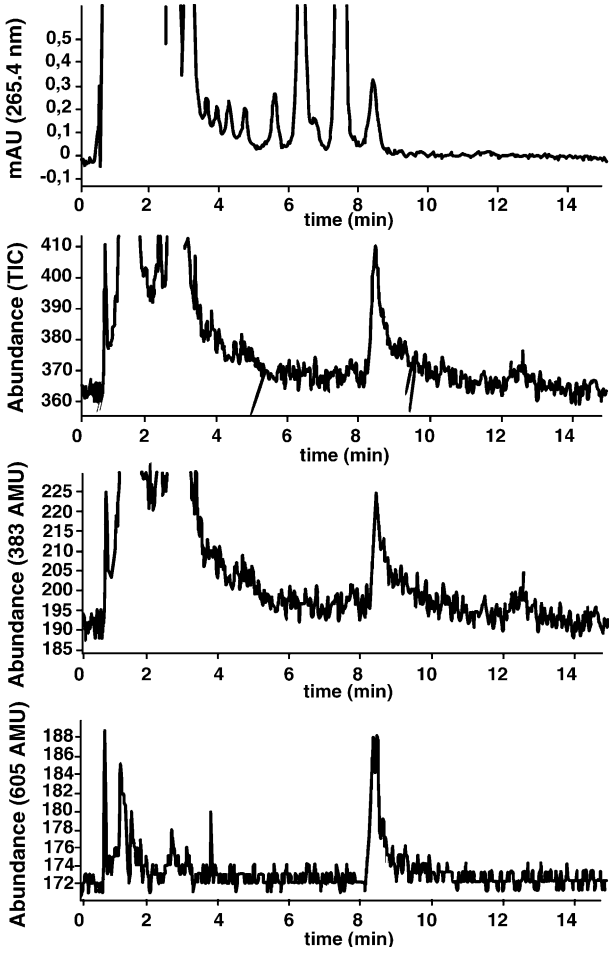

Fig. 3. HPLC–UV–MS detection of Fmoc-MML originated from plasma of the rat treated with L-deprenyl. Chromatographic conditions and monitoring as detailed for Fig. 1.

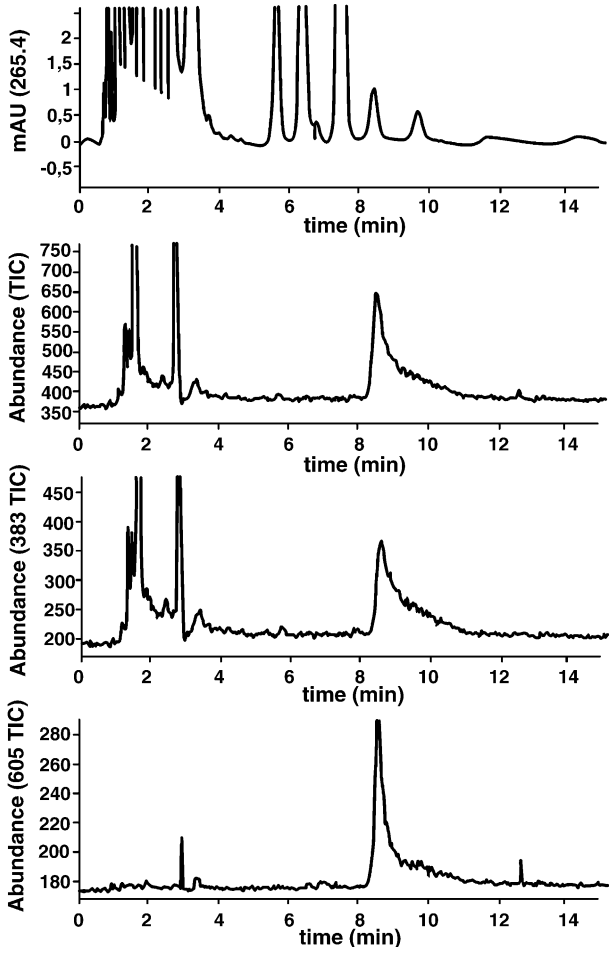

Fig. 4. HPLC–UV–MS detection of Fmoc-MML originated from liver of the rat treated with L-deprenyl. Chromatographic conditions and monitoring as detailed for Fig. 1.

the appearance of radiolabelled MML in the urine sample must have a long tailing that we have never found. The gross amount of radiolabelled MML was essentially eliminated in 6 h.

Metabolites are considered as the compounds resulting from a foreign compound in the body after having been subjected to alterations of its structure. The gross changes through metabolism are called metabolic pathways. The predicted metabolic reactions are detailed in the basic books of pharmacology, or they can be in silico (computer-modeled metabolism) calculated [8]. At the same time, the real method of finding the metabolic products is chromatography.

Neuroprotection supports the theory that the metabolites may have an essential role in therapy using L-deprenyl. Even the exceptionally low concentration of L-deprenyl has a dose-dependent influence on cell proliferation, as stimulation/inhibition on the apoptosis was found from 10^{-3} to 10^{-13} M concentration [14,15]. This phenomenon cannot be explained by a direct action of 10^{-13} M, however, peculiar metabolites may be responsible.

Nor-deprenyl, amphetamine, methamphetamine, *p*-hydroxy-methamphetamine, phenylacetone and several other compounds have been detected as metabolites of L-deprenyl. Recently Tábi et al. [11] separated the chiral isomers of tentative and existing metabolites of L-deprenyl using capillary zone electrophoresis, and no change of the chirality was found. Katagi et al. [2] found deprenyl-*N*-oxide as a major deprenyl metabolite subjected to urinary excretion.

To scout a trace level of metabolites, the radiolabelled parent compound has to be administered. Moreover, hyphenated methods such as HPLC–MS should be employed to prove the chemical structure of the metabolite present in a radioactive fraction.

The fate of the *N*-methyl group of L-deprenyl can be specified by several different metabolic pathways. An essential route is urinary elimination of the unchanged L-deprenyl, as well as of L-deprenyl-*N*-oxide, L-methamphetamine, and also formaldehyde. If *N*-demethylation resulted in a transmethylation to an amino acid (lysine) N^{ε}-monomethyllysine will also be detected. Methylation of RNA, DNA, and their

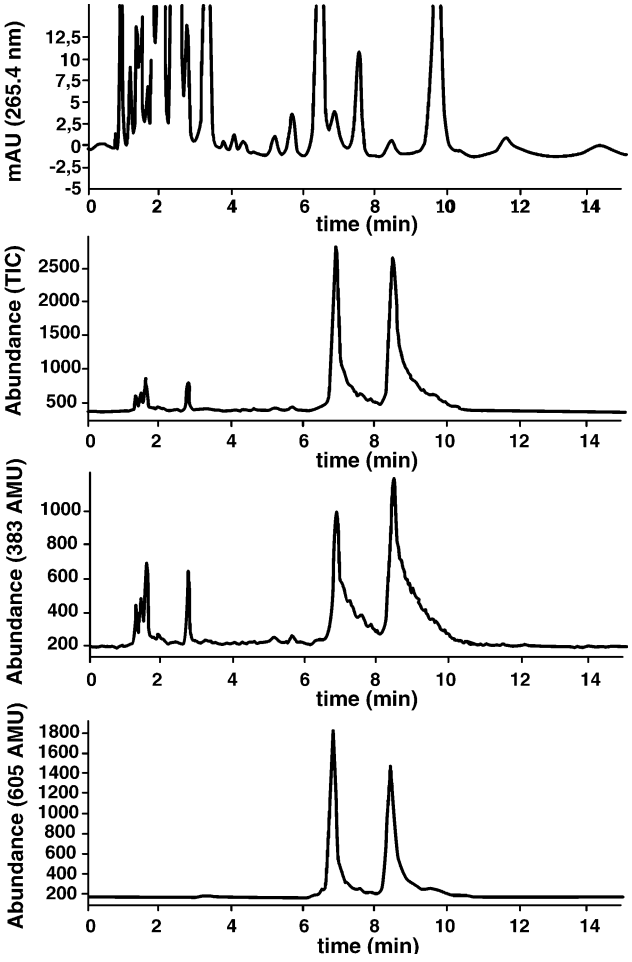

Fig. 5. HPLC–UV–MS detection of Fmoc-MML originated from brain of the rat treated with L-deprenyl. Chromatographic conditions and monitoring as detailed for Fig. 1.

fragments can also take place [16]. Some other alternative routes can also be postulated, such as elimination of metabolic fragment(s) through the lung. Demethylation yields formaldehyde, which is further oxidized through formic acid to carbon dioxide, and the carbon dioxide is eliminated with the exhausted air. The oxidation can be catalyzed by catalase enzyme, and a parallel reaction is decomposition of hydrogen peroxide to water. *N*-Demethylation metabolism can be thereby considered as a beneficial way to counterbalance the oxidative stress.

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