Arch Toxicol (1992) 66: 675–678

Archives of
Toxicology



*Short communication*

# Toxicological detection of selegiline and its metabolites in urine using fluorescence polarization immunoassay (FPIA) and gas chromatography-mass spectrometry (GC-MS) and differentiation by enantioselective GC-MS of the intake of selegiline from abuse of methamphetamine or amphetamine

**Hans H. Maurer and Thomas Kraemer**

Abteilung Toxikologie des Instituts für Pharmakologie und Toxikologie der Universität des Saarlandes, W-6650 Homburg (Saar), Federal Republic of Germany

Received 28 April 1992/Accepted 16 July 1992

**Abstract.** Selegiline (*R*(–)-N-methyl-N-(1-phenyl-2-propyl)-2-propinylamine), a selective MAO-B inhibitor used as an antiparkinsonian, is excreted in urine as N-desmethyl selegiline (norselegiline), *R*(–)-methamphetamine (*R*(–)-MA), *R*(–)-amphetamine (*R*(–)-AM) and their conjugated p-hydroxy derivatives. We found that the fluorescence polarization immunoassays (FPIA) TDx amphetamine/methamphetamine II (AM/MA II) and TDx amphetamine class (AM class) lead to positive results for up to 2 days after a single oral dose of 10 mg selegiline (detection limit: 0.1 mg/l, each). Every urine specimen from long term selegiline patients (10 mg/day) showed positive TDx results during the selegiline regimen. Positive TDx results were confirmed using gas chromatography-mass spectrometry (GC-MS). Selegiline metabolites, particularly MA, could be detected in urine for up to 7 days after intake of a single oral dose of 10 mg selegiline (detection limit: 0.01 mg/l for MA and AM). Norselegiline, the only specific selegiline metabolite, was only detectable for about 12 h. Moreover, norselegiline was not detected in all urine samples from long term selegiline patients (10 mg/day). Since differentiation of selegiline intake from MA/AM abuse by detecting norselegiline was not possible in most cases, an enantioselective GC-MS procedure was developed. It allowed differentiation of the enantiomers of the selegiline metabolites and thereby separation of selegiline intake (only *R*(–)-enantiomers) from methamphetamine and/or amphetamine abuse (racemates or *S*(+)-enantiomers). After derivatization with *S*(–)-N-trifluoroacetyl-prolyl chloride (TPC), the two enantiomers of MA and AM were each separated as diastereomers employing the routinely used achiral GC capillary. To prove the enantiomeric identity of MA and AM, before extraction, their *R*(–)-enantiomers were added to the urine samples which were tested positive in the TDx. Presence of *S*(+)-enantiomers of MA or AM revealed MA or AM abuse, whereas with selegiline intake only the *R*(–)-enantiomers of MA and/or AM were found. This enantioselective GC-MS procedure was sensitive enough to confirm all positive TDx results (detection limit: 0.1 mg/l for MA and AM).

**Key words:** Selegiline – Amphetamine – Methamphetamine – Metabolites – Fluorescence polarization immuno assay – Enantioselective gas chromatography-mass spectrometry – Analytical toxicology

## Introduction

Selegiline (*R*(–)-N-methyl-N-(1-phenyl-2-propyl)-2-propinylamine; Eldepryl™, Jumex™, Movergan™, Deprenyl™) is a selective MAO-B inhibitor, which is widely used as an antiparkinsonian. Recently, selegiline has been used monotherapeutically in the early phase of parkinsonism. Reynolds et al. (1978) showed that selegiline is metabolized to N-desmethyl selegiline (norselegiline), *R*(–)-methamphetamine (*R*(–)-MA), *R*(–)-amphetamine (*R*(–)-AM) and their conjugated p-hydroxy derivatives. The *R*(–)-enantiomers of AM and MA have five times less psychostimulant activity than the *S*(+)-enantiomers (Csanda et al. 1978). Since the *S*(+)-enantiomers or the racemates of AM and MA are misused, they have to be identified during a drug screening in clinicial and forensic toxicology as well as in doping control. Usually prescreening is performed using immunoassays. However, positive results must be confirmed by a second method, preferably GC-MS, to avoid misinterpretation. The Abbott fluorescence polarization immunoassays (FPIA) TDx

Dedicated to Prof. Dr. rer. nat. Dr. med. Ernst Mutschler, Frankfurt/Main, on the occasion of his 60th birthday.

Some of these results were reported at the 29th International Meeting of the TIAFT, Copenhagen, June, 24–27, 1991 (Maurer and Kraemer 1991 a) and at the Common Reunion of the French and German Pharmaceutical Societies, Strasbourg, September, 19–22, 1991 (Maurer and Kraemer 1991 b).

*Correspondence to:* H. H. Maurer

assays amphetamine/methamphetamine II and amphetamine class interfered with the *R*(–)-enantiomers of MA and AM (Fitzgerald et al. 1988), which are metabolites of the non-scheduled selegiline. A GC-MS procedure is presented to confirm the presence of selegiline metabolites in urine. In addition, we developed an enantioselective GC-MS procedure to differentiate the intake of selegiline from an abuse of MA/AM. Furthermore, the duration of detectability of the selegiline metabolites using FPIA and the two presented GC-MS methods is documented.

## Materials and methods

### *Apparatus*

A Hewlett-Packard (HP) series 5890 gas chromatograph combined with a HP MSD series 5970 mass spectrometer and a HP MS ChemStation (DOS series) with HP G1034B software were used. The GC conditions were as follows: splitless injection mode; column, HP capillary (12 m × 0.2 mm I. D.), cross-linked methylsilicone, 330 nm film thickness; injection port temperature, 270° C, carrier gas, helium; flow rate 1 ml/min.

*Non-enantioselective procedure.* Column temperature, programmed from 100 to 310° C in 30° C/min, initial time 3 min, final time 5 min.

*Enantioselective method.* Column temperature, programmed from 100 to 190° C in 30° C/min, initial time 3 min, final time 20 min.

The MS conditions were as follows: scan mode; ionization energy, 70 eV; ion source temperature, 220° C; capillary direct interface heated at 260° C.

### *Urine samples*

After being informed according to the declaration of Helsinki, three healthy volunteers received a single oral dose of 10 mg selegiline (*R*(–)-N-methyl-N-(1-phenyl-2-propyl)-2-propinylamine; Movergan™, Asta Pharma, Frankfurt/M., FRG). The urine samples were collected for 8 days and stored at –20° C before analysis. Blank urine samples were collected before the application of the drug, in order to control whether the samples were free of interfering compounds. Furthermore, urine samples were obtained from long term selegiline patients (10 mg/days).

### *Chemicals*

*S*(–)-N-trifluoroacetyl-prolyl chloride (TPC; 0.1 mol/l in dichloromethane) was purchased from ICT, Frankfurt/M, FRG. All other chemicals were obtained from E. Merck, Darmstadt, FRG.

### *Sample preparation for the non-enantioselective GC-MS procedure*

To a 10 ml volume of urine 1 ml of aqueous sodium hydroxide (1 mol/l) was added. The alkaline solution was extracted twice with a 10 ml portion of a mixture of ethyl acetate and diethyl ether (1 : 1). After phase separation by centrifugation the organic layer was transferred into a pear-shaped flask. Before evaporation, 0.1 ml of a mixture of three parts acetic acid anhydride and two parts pyridine was placed into the flask. Thereafter, the organic extract was carefully evaporated to dryness. A 0.1 ml aliquot of the acetylation mixture was added to the residue and kept at 60° C for 30 min. The acetylation mixture was then carefully evaporated to dryness and the residue dissolved in 0.1 ml methanol. A 1 µl volume of this sample was injected into the gas chromatograph.

### *Sample preparation for the enantioselective GC-MS procedure*

To a 10 ml volume of urine aliquots of *R*(–)-amphetamine and *R*(–)-methamphetamine were added to obtain a concentration which corresponded to 50% of the nominal concentration given by the TDx. The sample was alkalinized with 1 ml aqueous sodium hydroxide (1 mol/l) and then extracted twice with a 10 ml portion of a mixture of ethyl acetate and diethyl ether (1 : 1). After phase separation by centrifugation the organic layer was transferred into a pear-shaped flask. Before evaporation, 1 ml of a 0.1 mol/l solution of *S*(–)-N-trifluoroacetyl-prolyl chloride (TPC) in dichloromethane was placed in the flask. Thereafter, the mixture was carefully evaporated to dryness and the resultant residue dissolved in 0.1 ml dichloromethane. A 1 µl volume of this sample was injected into the gas chromatograph.

### *Gas chromatographic-mass spectrometric analysis*

*Non-enantioselective method.* Mass chromatography with masses m/z 58, 82 and 86 was used to indicate the presence of acetylated N-desmethyl selegiline (82), amphetamine (86) and methamphetamine (58).

*Enantioselective method.* Mass chromatography with masses m/z 237 and 251 was used to indicate the presence of TPC-derivatized amphetamine (237) and TPC-derivatized methamphetamine (251).

For both methods the peak underlying mass spectra were identified by computer library search (Pfleger et al. 1992).

### *Immunoassays*

The Abbott TDx assays amphetamine/methamphetamine II (AM/MA II) and amphetamine class (AM class) were used for the immunological determination of native urine samples. The cross reactivities at a concentration level of 1 mg/l were as follows: AM/MA II: 36% for the *R*(–)-enantiomer of amphetamine, AM class: 40% for the *R*(–)-enantiomer of amphetamine and 85% for *R*(–)-methamphetamine. The cut-off was: 0.3 mg/l for the AM/MA II assay and 0.5 mg/l for the AM class assay. The detection limit was 0.1 mg/l for both assays.

## Results and discussion

### *Sample preparation*

For extraction of the amines alkaline pH was adjusted with aqueous sodium hydroxide. To prevent the volatile amines from evaporating, the acetylation mixture was placed into the tube before evaporation of the extraction mixture to form acetyl derivatives, which are less volatile. A subsequent acetylation for 30 min was indispensable for a complete derivatization. Derivatization with the chiral reagent TPC lead to diastereomers, which were separable on the routinely used achiral GC column. We found that careful evaporation of the mixture of organic extract and TPC over several minutes is sufficient for derivatization. A longer reaction time did not lead to a better result.

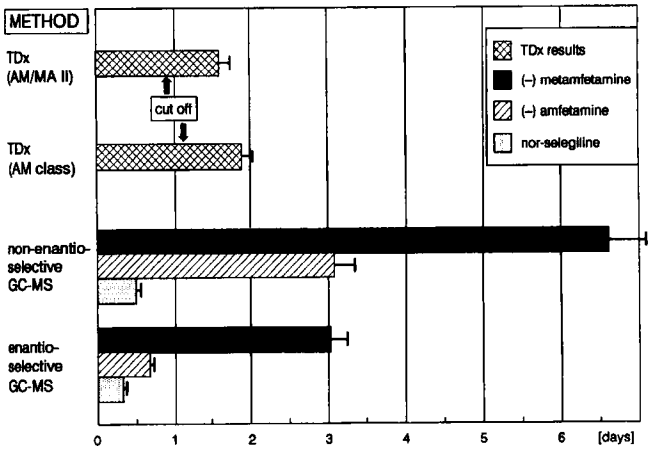


**Fig. 1.** Duration of detectability of selegiline metabolites in urine ($n = 3$) by different methods after intake of 10 mg selegiline

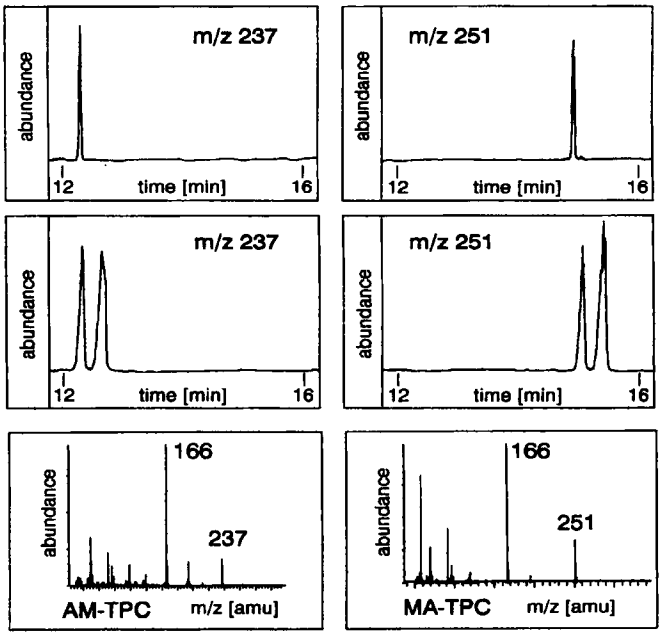


**Fig. 2.** Differentiation of the intake of selegiline from methamphetamine and/or amphetamine abuse using enantioselective GC-MS. The top part of the figure shows mass chromatograms with the masses m/z 237 for TPC derivatized amphetamine (AM) (*left side*) and m/z 251 for TPC derivatized methamphetamine (MA) (*right side*). The appearance of only one peak for each diagnostic mass indicates the intake of selegiline (only *R*(−)-enantiomers). The appearence of two peaks for every diagnostic mass indicates methamphetamine and/or amphetamine abuse (racemic substance or *S*(+)-enantiomer plus *R*(−)-enantiomer added as control to the urine samples). The bottom portion of this figure shows the mass spectra of TPC derivatized amphetamine (AM-TPC) and TPC derivatized methamphetamine (MA-TPC) with the base peak of m/z 166 each and the diagnostic ions m/z 237 and 251. The mass spectra are suitable for proving the identity of the peaks

*Detection by FPIA*

The results of our studies are summarized in Fig. 1. After ingestion of a single oral dose of 10 mg selegiline, the TDx assays amphetamine/methamphetamine II and amphetamine class gave positive results for about 1 day, using the cut-off recommended by the manufacturer, and even for about 2 days when the detection limits were taken into consideration.

*Detection by non-enantioselective GC-MS*

Using mass chromatography with the masses m/z 58, 82 and 86, the presence of acetylated N-desmethyl selegiline (82), amphetamine (86) and methamphetamine (58) was indicated. The peak underlying mass spectra were identified by computer library search (Pfleger et al. 1992). As shown in Fig. 1, selegiline metabolites, particularly methamphetamine, could be detected by this GC-MS method for about 7 days after intake of a single oral dose of 10 mg of selegiline (detection limit: 0.01mg/l for amphetamine as well as for methamphetamine). The latter substances lead to positive TDx results for up to 2 days, whereas the selegiline specific metabolite norselegiline was detectable by GC-MS for only about 12 h. Even in the urine samples of long term selegiline patients (10 mg/day) norselegiline could not be detected in all cases. Therefore, differentiation of the intake of selegiline from AM/MA abuse by non-enantioselective GC-MS was not always possible. Differentiation of the intake of the drugs must be made by detecting the two enantiomers of amphetamine and methamphetamine.

*Detection by enantioselective GC-MS*

Mass chromatography with masses m/z 237 and 251 indicated the presence of TPC-derivatized amphetamine and TPC-derivatized methamphetamine: S-(−)-trifluoroacetyl-prolyl-*R,S*-amphetamine (237), *S*-(−)-N-trifluoroacetyl-prolyl-*R,S*-methamphetamine (251). The peak underlying mass spectra were identified by computer library search (Pfleger et al. 1992). Applying this GC-MS method, selegiline metabolites, particularly methamphetamine, could be detected for about 3 days after intake of a single oral dose of 10 mg selegiline (detection limit: 0.1 mg/l for amphetamine as well as for methamphetamine).

*Differentiation of the intake of selegiline from methamphetamine and/or amphetamine abuse by enantioselective GC-MS*

For differentiation we detected the two enantiomers of amphetamine and methamphetamine. The gas chromatographic separation of the enantiomers of AM and MA on an achiral GC column was achieved by converting them into diastereomers. This conversion was performed with the chiral reagent *S*(−)-TPC. We preferred this method, since the amphetamines must be derivatized in any case and since the routinely used achiral column need not be changed. To prove the enantiomeric identity of AM and MA, before extraction, their *R*(−)-enantiomers were added as controls to the urine samples which tested positive in the

TDx. In our experience, it is practical to add an amount of the *R*(–)-enantiomers which leads to a urine concentration corresponding to about 50% of the nominal concentration given by the TDx. As shown in Fig. 2, in the case of selegiline intake only one peak appeared in the mass chromatograms (m/z 237 and 251) for AM and MA, respectively (*R*(–)-enantiomers). In case of intake of racemic AM/MA or of the *S*(+)-enantiomers two peaks each appeared in the mass chromatograms representing the two diastereomers of the TPC derivatives. Since this enantioselective method had the same detection limit (0.1 mg/l) as the TDx assays for AM and MA, it could expose AM/MA abuse, as indicated by the FPIA. Moreover, this procedure allowed the differentiation of such an abuse from selegiline intake. Proving the *S*(+)-enantiomers of amphetamine and methamphetamine to be absent in the urine specimen is sufficient to clear a selegiline patient from the suspicion of methamphetamine and/or amphetamine abuse. If the *S*(+)-enantiomers of amphetamine or methamphetamine are present in a specimen, an abuser's protestation of having taken only the non-scheduled selegiline can be disproved. In the meantime the enantioselective procedure has been successfully applied in many clinical cases.

## Conclusions

In clinical and forensic toxicology as well as in doping control every immunoassay result must be confirmed by a second method. The non-enantioselective GC-MS procedure presented in this paper confirmed every positive result of the TDx assays for AM and MA, because it was much more sensitive than the TDx assays. However, differentiation of selegiline intake from MA/AM abuse by detecting the only specific selegiline metabolite, norselegiline, was not possible in every case, because norselegiline is only a minor metabolite of selegiline and could not be detected in urine as long as the TDx assays gave positive results.

The enantioselective GC-MS procedure allowed the differentiation of the intake of selegiline from abuse of AM and MA, since selegiline and its metabolites AM and MA are *R*(–)-enantiomers whereas the *S*(+)-enantiomers of AM and MA or their racemates are misused as psychostimulants. Therefore, the enantioselective procedure is indicated in cases of doubt in clinical and forensic toxicology to avoid misinterpretation of positive TDx results and their GC-MS confirmation.

*Acknowledgements.* The authors wish to thank Dr. H. Jäger for providing urine specimens from long term selegiline patients and the Bundesminister für Jugend, Familie, Frauen und Gesundheit for providing instruments.

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