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Detection, Quantification, Metabolism, and Behavioral Effects of Selegiline in Horses*

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ABSTRACT

Selegiline ([R]-[-]*N*,α-dimethyl-*N*-2-propynylphenethylamine or *l*-deprenyl), an irreversible inhibitor of monoamine oxidase, is a classic antidyskinetic and antiparkinsonian agent widely used in human medicine both as monotherapy and as an adjunct to levodopa therapy. Selegiline is classified by the Association of Racing Commissioners International (ARCI) as a class 2 agent, and is considered to have high abuse potential in racing horses. A highly sensitive LC/MS/MS quantitative analytical method has been developed for selegiline and its potential metabolites amphetamine and methamphetamine using commercially available deuterated analogs of these compounds as internal standards. After administering 40 mg of selegiline orally to two horses, relatively low (<60 ng/ml) concentrations of parent selegiline, amphetamine, and methamphetamine were recovered in urine samples. However, relatively high urinary concentrations of another selegiline metabolite were found, tentatively identified as *N*-desmethylselegiline. This metabolite was synthesized and found to be indistinguishable from the new metabolite recovered from horse urine, thereby confirming the chemical identity of the equine metabolite. Additionally, analysis of urine samples from four horses

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dosed with 50 mg of selegiline confirmed that *N*-desmethylselegiline is the major urinary metabolite of selegiline in horses. In related behavior studies, PO and IV administration of 30 mg of selegiline produced no significant changes in either locomotor activities or heart rates.

■ INTRODUCTION

Selegiline ([R]-[-]*N*,α-dimethyl-*N*-2-propynylphenethylamine or *l*-deprenyl) is a classic antiparkinsonian drug widely used in human medicine.[1] Selegiline is used in parkinsonian patients both as monotherapy and as an adjunct to levodopa therapy. Selegiline and its metabolite *N*-desmethylselegiline are irreversible inhibitors of monoamine oxidase type B (MAO-B), and they inhibit the metabolism of dopamine in the human brain.[2] Additionally, by inhibiting the ATP–sensitive potassium channels (K_{ATP}), selegiline and its metabolite methamphetamine induce dopamine release in the rat caudate–putamen in vitro.[3] The ability of selegiline to inhibit both the K_{ATP} channels and MAO-B enzymes makes this compound a suitable agent for use as a catecholaminergic activity enhancer. Therefore, selegiline has the potential to be abused in racing horses as an illegal aid to increase their performance. According to the Association of Racing Commissioners International (ARCI), it is a class 2 agent. Therefore, there is a need for effective screening and validated confirmation methods to enable control of its use during equine drug testing.

The major urinary metabolites of selegiline in humans and laboratory animals include methamphetamine, amphetamine, and *N*-desmethylselegiline,[4] respectively (Figure 1). Both methamphetamine and *N*-desmethylselegiline are further metabolized to amphetamine (Figure 1). The oral bioavailability of selegiline is poor and its plasma half-life is short,[5] suggesting that highly sensitive analytical methods are required for the detection and confirmation of this compound in biologic fluids of horses. Additionally, there are no reported studies on the pharmacologic activity and pharmacokinetics of selegiline or its metabolites in horses. The main objective of this study was to develop a practical highly sensitive analytical method for detection, confirmation, and quantification of selegiline and its metabolites in the biologic fluids of horses.

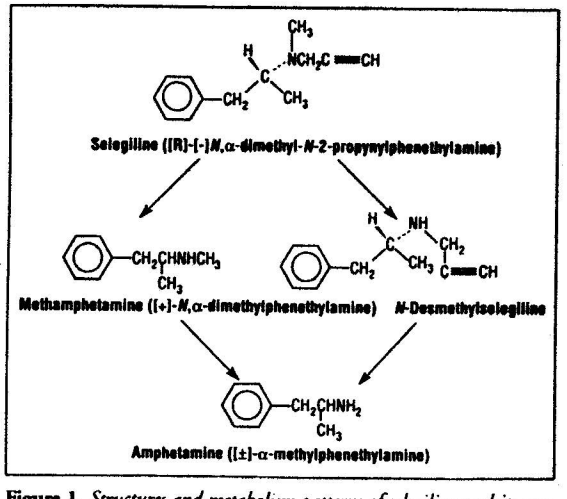

Figure 1. *Structures and metabolism patterns of selegiline and its major urinary metabolites in humans and laboratory animals.*

■ MATERIALS AND METHODS

Animals and Treatments

Two mature thoroughbred mares (461 and 555 kg) were used for the preliminary study. Selegiline hydrochloride was obtained in 5-mg tablets (Eldepryl, ESI Lederle) and administered PO as a single dose (40 mg).

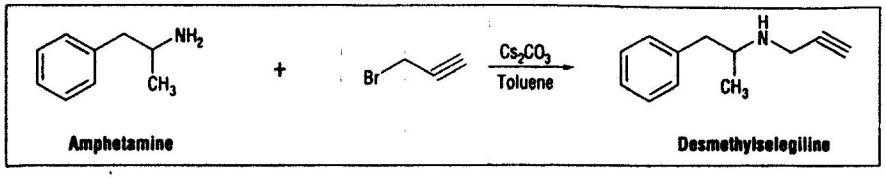

Figure 2. *Synthesis of* N-*desmethylselegiline from amphetamine.*

In the second series of evaluations, a single dose (50 mg) was administered to four mature thoroughbred mares. Pooled urine samples were collected from these horses with a Foley catheter and attached plastic bag, separated into aliquots, and stored at –20°C until they were assayed.

For locomotor chamber studies, 30 mg of selegiline hydrochloride was administered PO and IV to two mature thoroughbred mares in a crossover experiment in which each horse served as its own control. The oral bioavailability of selegiline in other animal species is very poor, and it is not known in horses; therefore, for the locomotor chamber studies, selegiline was administered by the IV route in addition to PO dosing. For PO dosing, selegiline hydrochloride was obtained as 5-mg tablets, and an injectable formulation was acquired from the United States Pharmacopeia (USP) in Rockville, MD for IV use. All animal care was in compliance with the guidelines issued by the Division of Laboratory Animal Resources and approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

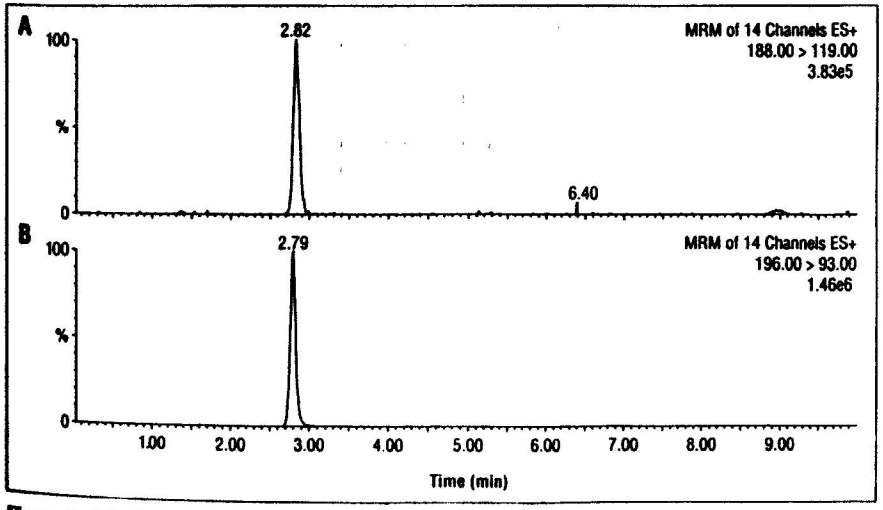

Figure 3. *Selegiline chromatography in mobile phase: transition* m/z *188→119 for selegiline (retention time = 2.82 minutes)* (A), m/z *196→93 for selegiline-d_8 (retention time = 2.79 minutes)* (B).

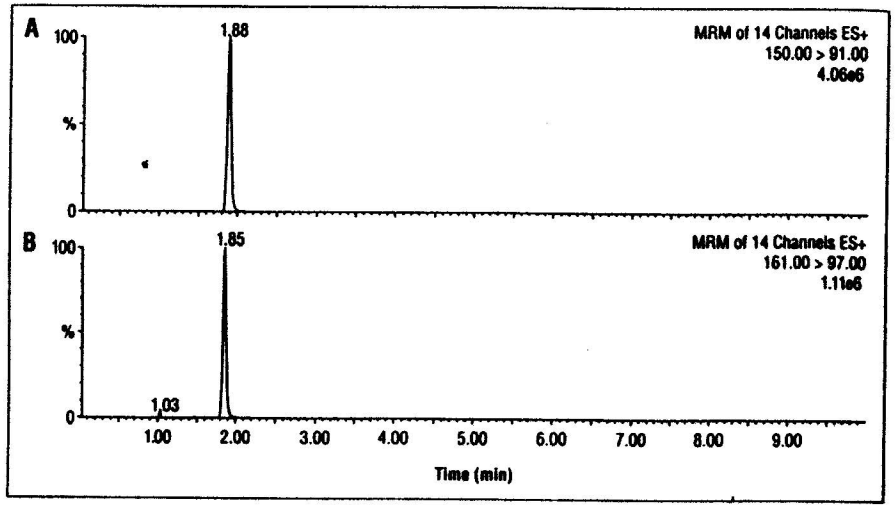

Figure 4. *Methamphetamine chromatography in mobile phase: transition* m/z *150→91 for methamphetamine (retention time = 1.88 minutes)* **(A)**, m/z *161→97 for methamphetamine-d_{11} (retention time = 1.85 minutes)* **(B)**.

Liquid/Liquid Extraction Method

Stock standards (1 mg/ml) of selegiline hydrochloride, methamphetamine hydrochloride, amphetamine sulfate, and *N*-desmethylselegiline were prepared in acetonitrile with traces of water added where necessary to assure full solubility of salts. All standards were acquired from Sigma, except *N*-desmethylselegiline, which was synthesized as described below. Dilutions of stock standards were made using 85% solvent A (5% acetonitrile and 0.05% formic acid in water) and 15% solvent B (0.05% formic acid in acetonitrile). Internal standards (selegiline-d_8, amphetamine-d_8, and methamphetamine-d_{11}) (Sigma) were dissolved in methanol (100 µg/ml) and were diluted to 1% in methanol to yield standard solutions of 1 ng/µl for analysis. To each 1-ml sample, 25 µl of internal standard (1 ng/µl) was added. Standards were prepared by the addition of specific amounts of selegiline and the metabolites dissolved in 85% solvent A and 15% solvent B to blank urine samples (1 ml each) over a range from 50 pg/ml to 50 ng/ml (i.e., 0.05, 0.1, 1, 10, 25, 50 ng/ml).

Extraction Method

A liquid/liquid extraction recovery method was used for selegiline and its primary metabolites in equine biologic samples. An aliquot (1 ml) of urine sample was transferred to a 20-ml culture tube equipped with a Teflon (DuPont)-lined screw cap. Sample pH was increased to greater than 10 by adding 1 ml of potassium carbonate–saturated distilled water. The basic mixture was extracted with 3 ml of dichloromethane (DCM) by gently mixing the contents for 15 minutes using a motorized rotorack. The mixture was centrifuged at 1000 × *g* at 4°C for 5 minutes in a swinging bucket rotor centrifuge (Beckman). The top aqueous layer was transferred by pipette into new culture tubes, and 2 ml DCM was added and mixed on the motorized rotorack for 15 minutes, centrifuged at 1000 × *g* at 4°C for 5 minutes, and

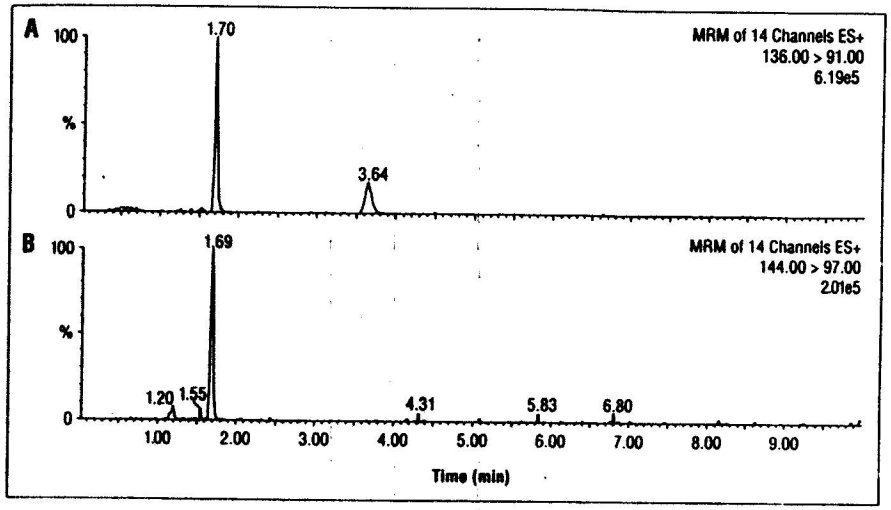

Figure 5. *Amphetamine chromatography in mobile phase: transition* m/z *136→91 for amphetamine (retention time = 1.70 minutes)* **(A)**, m/z *144→97 for amphetamine-d_8 (retention time = 1.69 minutes)* **(B)**.

the top aqueous layer was aspirated to waste. The organic layers (bottom layer) from the first and second extractions were combined and transferred to labeled dimethyldichlorosilane (Pierce Biotechnology)–silanized taper-bottom tubes. Acidified methanol (100 µl, 4% v/v of concentrated hydrochloric acid in methanol) was added to the DCM mixture prior to evaporation. The solvent was evaporated under a stream of nitrogen gas at 37°C. The residue was resuspended in 100 µl of a mixture of 85% solvent A and 15% solvent B with moderately vigorous vortexing. This solution was placed into an autosampler vial containing a 200-µl insert, and 40 µl of this solution was injected into the LC/MS/MS for analysis.

Synthesis of *N*-desmethylselegiline

N-desmethylselegiline (2-methyl-*N*-2-propynylphenethylamine) was obtained from amphetamine sulfate after alkylation with propargyl bromide (Figure 2).[6] First, amphetamine sulfate was converted into the free amine by treating the compound with sodium ethanolate in absolute ethanol. Precipitated sodium sulfate was filtered off; ethanol was evaporated, and free amphetamine was dissolved in toluene. To this solution, cesium carbonate and a molar equivalent of propargyl bromide was added. The reaction mixture was stirred overnight at room temperature. The alkylation resulted in a mixture of *N*-desmethylselegiline and *N,N*-dipropargylamphetamine in a ratio of approximately 3:1. The reaction mixture was filtrated through celite, toluene was evaporated under reduced pressure, and the mixture was separated on a silica gel column using acetone–hexane (1:1) as eluent. Pure *N*-desmethylselegiline was obtained as a colorless oil.

Synthetic *N*-desmethylselegiline was assessed for proper molecular weight, structure, and purity by dissolution underivatized in methanol, followed by analysis by gas chromatography (GC)/MS (Agilent 6890/5972, 30 m ×

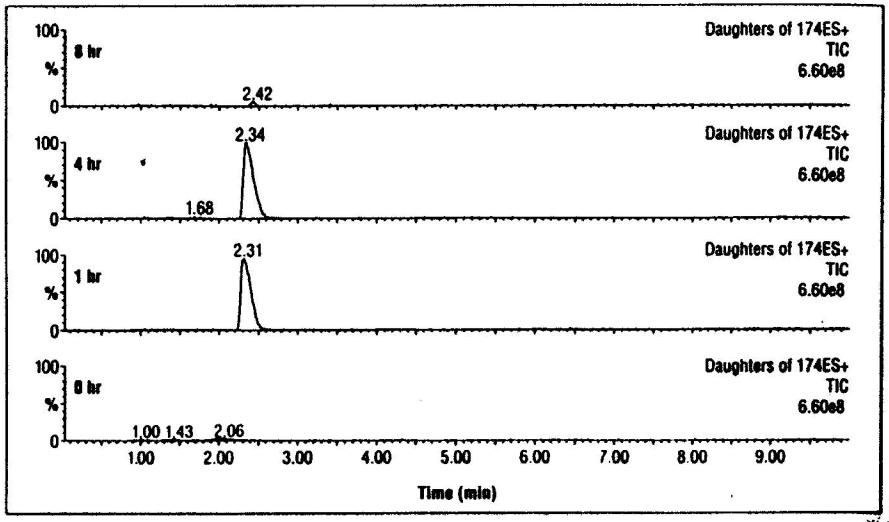

Figure 6. *Daughter ion chromatograms for urine extracts 0,1,4, and 8 hours after administration, with specific screening for* N*-desmethylselegiline, showing a predicted* m/z *of 174 for its* $[M+H]^+$ *molecular ion.*

0.25 mm × 0.25-μ film HP-5MS column, 70°C [held 1 minute], 20°C per minute to 280°C [held 12 minutes], helium flow 1 ml per minute). The resultant 8-minute retention-time peak had a corresponding mass spectrum that showed a very meager molecular ion at *m/z* 173, with loss of methyl to give a more obvious *m/z* 158. Principal peaks in order of abundance included *m/z* 82 (base), 91, 65, 55, 115, 128, and 158. The spectrum was successfully deconvoluted with MassSpec Calculator Pro software (Quadtech Associates). The compound was further examined by direct infusion ESI(+)-MS/MS (dissolved to 10 μg/ml in acetonitrile:0.05% formic acid [aqueous], 1:1) and shown to bear a distinct [M+H] peak at *m/z* 174, with isotopic peaks matching those expected for a $C_{12}H_{16}N$ compound. Principal daughter ion peaks in order of abundance included *m/z* 91, 56, 119, 39, 41, and 65, which were also interpretable by Calc Pro (Microsoft) software. Peak purity by GC/MS appeared to be 99% or greater.

Instrumentation and Analysis

The Quattro II Electrospray Ionization Tandem Quadropole Mass Spectrometer (Micromass) was tuned for optimum sensitivity by direct infusion of a mixture of 1 μg/ml amphetamine, methamphetamine, and selegiline in acetonitrile:0.05% formic acid, 1:1. Responses were maximized for the following transitions: amphetamine *m/z* 136→91; methamphetamine *m/z* 150→91; selegiline *m/z* 188→91. In all, 14 MS/MS transitions were monitored as follows, with dwell times (seconds) shown in parentheses: *m/z* 136→65 (0.01); *m/z* 136→119 (0.01); *m/z* 136→91 (0.03); *m/z* 144→97 (0.03); *m/z* 150→91 (0.03); *m/z* 150→65 (0.01); *m/z* 150→119 (0.01); *m/z* 161→97 (0.03); *m/z* 174→91 (0.01); *m/z* 174→119 (0.01); *m/z* 188→70 (0.01); *m/z* 188→119 (0.01); *m/z* 196→93 (0.03). Typical MS/MS tuning parameters are shown in Table 1.

TABLE 1. Tuning Parameters for MS/MS for Detection of Selegiline and Metabolites

ESI+ Source	
Capillary	3.10 kV
Cone	0.50 kV
Cone gas flow	20 V
Source offset	5 V
Source temperature	1.8 V
Desolvation temperature	100 V
MS1	120 °C
Ion energy	5.5 V
Ion energy 2	5.9 V
Lens 1	100 V
Lens 2	4 V
Multiplier	680 V
MS2	
Ion energy	4.6 V
Ion energy 2	250 V
Lens 1	270 V
Lens 2	
Multiplier	
Collision gas	2.5 mbar
Collision energy	20 V

HP1050 LC conditions (isocratic) consisted of 85% solvent A and 15% solvent B with a flow rate of 0.5 ml/min and a stop time of 10 minutes. Injector parameters were draw speed 200.0 μl/min; eject speed 200.0 μl/min; and draw position 0.00 mm.

The areas of the peaks corresponding to selegiline, *N*-desmethylselegiline, methamphetamine, amphetamine, and internal standards (selegiline-d_8, methamphetamine-d_{11}, amphetamine-d_8) were recorded, and the internal standard values were used to normalize the selegiline, *N*-desmethylselegiline, methamphetamine, and amphetamine areas. Integrated peak values were entered into QuattroPro for Windows (Corel) for statistical analysis of standards and for interpolation of unknown amounts of selegiline and metabolites. Standard curves were generated with SigmaPlot for Windows (SPSS). Samples yielding a signal response greater than that produced by the high standard were diluted as necessary and rerun.

Behavioral Studies

The locomotor chambers have been described previously.[7] Briefly, two 3.4 × 3.4–m box stalls were equipped with Minibeam sensors (SM31E and SM2A31R, Banner Engineering) spaced equally around the stall 45 cm above the floor. Each time the horse disrupted the beam of light, an interruption was scored, and this output was summed and recorded on a data logger (CR10, Campbell Scientific).

Heart rates were recorded at 1-minute intervals during each experiment by an on-board heart rate computer (Polar CIC). An elastic strap with a receiver and attached transmitter was placed around the chest of the horse. The transmitter was connected to two electrodes placed on shaved areas of the sternum and left side of the anterior chest. Electrode gel was used to ensure proper conduction of the signal.

Behavioral experiments followed a rigorous standard protocol to reduce variability from extraneous effects. Each horse was placed in a behavior stall at 7:00 AM, and the heart rate strap was attached. The horse was allowed to acclimate to the stall for 7 hours. Recording of locomotor and heart rate activities was begun at 2:00 PM. Baseline activity was recorded for 30 minutes, after which the experimental treatments were administered. Locomotor and heart rate data were recorded for 14 hours until 5:00 AM the following morning. The total number of interruptions was summed every 15 minutes.

TABLE 2. Interpretation of Primary Peaks in ESI(+) MS Daughter Ion Spectrum of the Putative N-desmethylselegiline Metabolite *N*-desmethylselegiline as Seen in Figure 7

m/z (m/e)	Interpretation
174	*N*-desmethylselegiline [M+H]+
119	Phenylpropyl fragment
91	Benzyl fragment
56	*N*,2-methylpropanamine fragment
41	5-Carbon alkenyl ring fragment
39	2-Propenyl fragment
30	Internal propyl fragment
28	3-Carbon alkenyl ring fragment
28	Internal ethyl fragment

■ RESULTS

The developed LC/MS/MS method is relatively sensitive, with 50 pg/ml in a sample volume of 1 ml determined as the limit of detection (LOD) for selegiline, amphetamine, methamphetamine, and *N*-desmethylselegiline. LOD was determined by measuring mean blank background values, calculating the standard deviation of such backgrounds, and adding two standard deviations to the mean value.[8]

The chromatogram of selegiline detected as a monoprotonated analog at 2.8 minutes retention time with the transition *m/z* 188→119 for selegiline and *m/z* 196→93 for selegiline-d_8 is shown in Figure 3. The chromatogram of methamphetamine detected at 1.9 minutes retention time as a monoprotonated analog of parent compound with transition *m/z* 150→91 for methamphetamine and *m/z* 161→97 for methamphetamine-d_{11} is shown in Figure 4. The chromatogram of amphetamine detected at 1.7 minutes retention time as a monoprotonated analog of the parent compound with the transition *m/z* 136→91 for amphetamine and *m/z* 144→97 for amphetamine-d_8 is displayed in Figure 5. The benzyl fragment of *m/z* 91 was the primary daughter ion for selegiline, methamphetamine, amphetamine, and *N*-desmethylselegiline.

Internal standard daughter ions were measured as follows in order of ion abundance: d_{11}-methamphetamine (M+H = *m/z* 161): *m/z* 161→97, *m/z* 161→127, *m/z* 161→36, *m/z* 161→64, *m/z* 161→44; d_8-selegiline (M+H = *m/z* 196): *m/z* 196→93, m/z 196→124, *m/z* 196→74, *m/z* 196→33; d_8-amphetamine (M+H = *m/z* 144): *m/z* 144→97, *m/z* 144→127, *m/z* 144→110, *m/z* 144→84, *m/z* 144→69, *m/z* 144→44.

Comparisons of chromatograms that specifically detect *m/z* 174 daughter ions from urine extracts 0, 1, 4, and 8 hours after drug administration are shown in Figure 6. This procedure enables specific screening for *N*-desmethylselegiline, with a predicted *m/z* of 174 for the $[M+H]^+$ molecular ion. Interpretation of the primary peaks in the ESI(+) MS daughter ion spectrum of selegiline's putative *N*-desmethylselegiline metabolite is shown in Table 2. To confirm the identity of the metabolite detected in urine samples, *N*-desmethylselegiline was synthesized from amphetamine sulfate. The daughter ion spectrum of synthetic *N*-desmethylselegiline was identical to the principal metabolite recovered from horse urine samples, thereby confirming the chemical identity of this metabolite (Figure 7). In preliminary work, the amount of *N*-desmethylselegiline in urine samples relative to selegiline-d_8 internal standard was estimated and calculated to be 400 ng/ml at 4 hours.

The standard curves were linear for selegiline, methamphetamine, and amphetamine from 50 pg/ml to 50 ng/ml as shown in Figure 8. The "extracted samples" are those that were obtained by liquid/liquid partitioning from blank urine spiked with standard solutions and DCM. Direct injection samples represent blank urine spiked with standards that were then passed through an Amikon Millipore filter with a cutoff of 3,000 D molecular weight without liquid/liquid extraction. The similarity of the curves indicated that there is no significant change in the relative response induced by the process of extraction. Preliminary work indicated that the major urinary metabolite of selegiline is likely to be *N*-desmethylselegiline followed by amphetamine and methamphetamine, respectively (Figure 9). Selegiline, as parent compound, was also detected in urine samples at a peak concentration of 9.7 ng/ml at 4 hours.

To confirm these results, *N*-desmethylselegiline was synthesized for quantification of this metabolite in equine biologic fluids. Using the *N*-desmethylselegiline standard and selegiline-d_8 as the internal standard, the concentrations of this metabolite in equine urine were determined. As shown in Figure 10, following single PO administration of 50 mg of selegiline, the major urinary metabolite was *N*-desmethylselegiline, peaking at 2 hours at a concentration of 480 ng/ml. Selegiline as a parent compound was not identified from pooled urine samples, and the second major urinary metabolite was found to be amphetamine, which

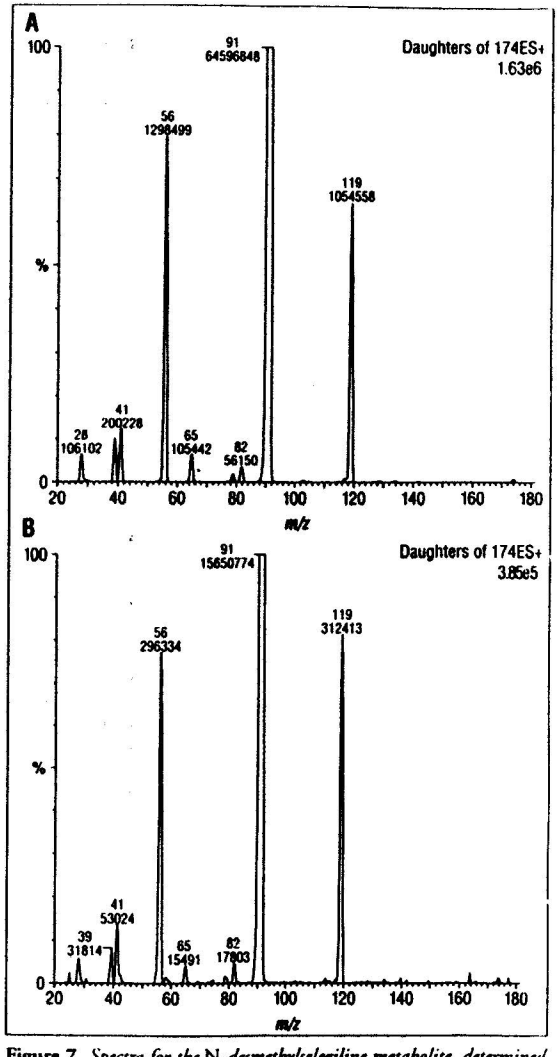

Figure 7. *Spectra for the* N*-desmethylselegiline metabolite, determined by daughter ion scan for* m/z *174. The daughter ion spectrum of synthesized* N*-desmethylselegiline* **(A)** *was identical to* N*-desmethylselegiline recovered from the urine sample tested 1 hour after administration of selegiline* **(B)**, *thereby confirming the chemical identity of this metabolite. The spectrum intensity has been expanded for clarity of minor peaks.*

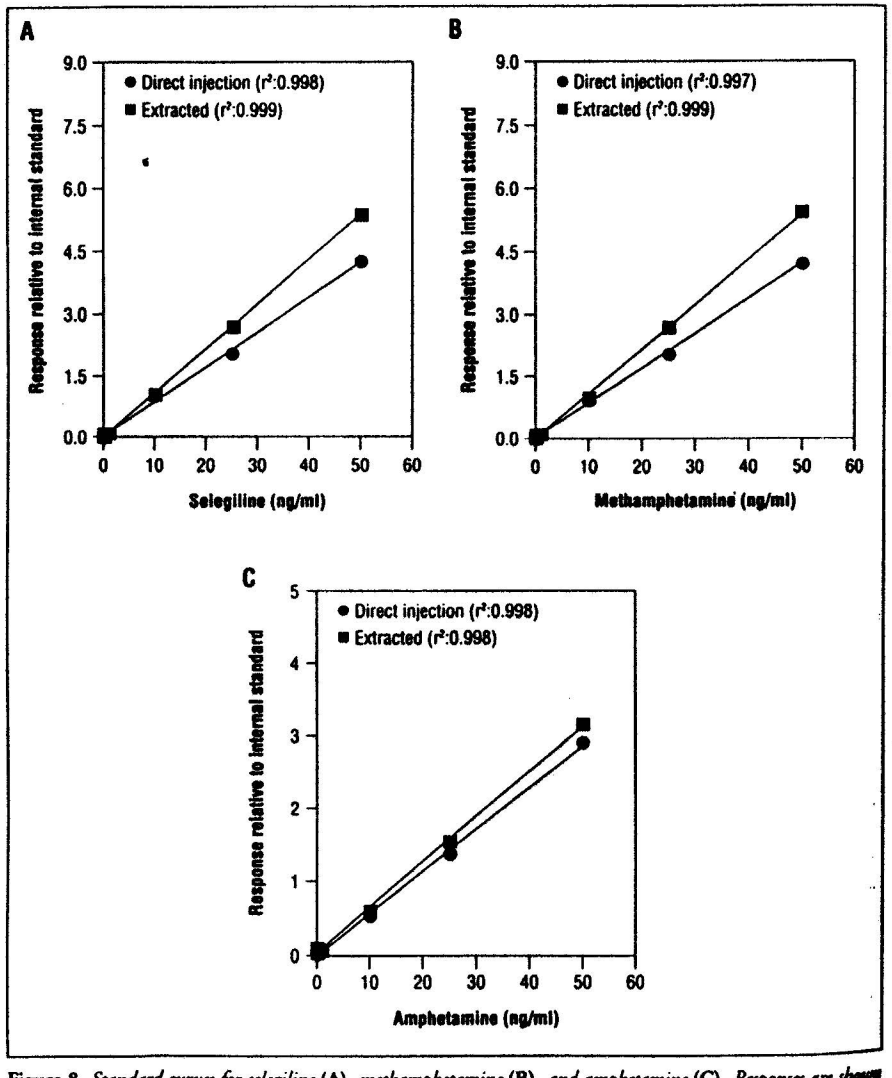

Figure 8. *Standard curves for selegiline* (A), *methamphetamine* (B), *and amphetamine* (C). *Responses are shown relative to selegiline-d_8, methamphetamine-d_{11}, and amphetamine-d_8, respectively. Extracted samples represent those that were extracted by liquid/liquid partitioning between blank urine spiked with standard solutions and dichloromethane. Direct injection samples represent blank urine spiked with standards that were then filtered (limit of 3,000 D molecular weight) without liquid/liquid extraction.*

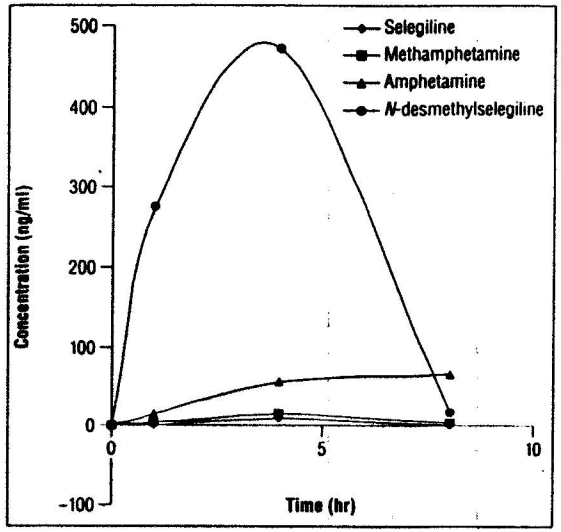

Figure 9. *Early time course for selegiline and its equine urinary metabolites (preliminary examination following administration of a single PO dose of 40 mg).* N-*desmethylselegiline concentrations were estimated by comparison with selegiline responses.*

was followed by methamphetamine. Peak urinary concentrations of amphetamine at 38 ng/ml were obtained at 24 hours after administration, and peak urinary concentrations of methamphetamine (6.1 ng/ml) were obtained at the 4-hour sampling. Only amphetamine was identified in these urine samples (0.41 ng/ml) at 24 hours after administration.

In the behavioral studies, there were no significant changes in either locomotor activities or heart rate following PO and IV administration of selegiline at 30 mg/kg, relative to values for untreated controls.

DISCUSSION

The primary objective of this study was to develop a highly sensitive analytical method for detection and quantification of selegiline and its major metabolites (amphetamine, methamphetamine, and *N*-desmethylselegiline) in the biologic fluid of horses. The LC/MS/MS method that was developed permitted detection of the parent compound and these metabolites, with the LOD being 50 pg/ml in a 1-ml sample.

The major urinary metabolites of selegiline in humans and laboratory animals are methamphetamine, followed by amphetamine and *N*-desmethylselegiline.[4] Based on the preliminary study described here, the major urinary metabolites of selegiline in equine species occur with the reverse relationship (i.e., *N*-desmethylselegiline followed by amphetamine and methamphetamine). Due to the unavailability of *N*-desmethylselegiline at the beginning of this study, standard solutions could not be generated for the accurate quantification of this metabolite in the urine samples of the horses. However, the relative amount of *N*-desmethylselegiline in urine samples was estimated relative to selegiline and its selegiline-d_8 internal standard from two horses dosed with 40 mg of selegiline and was later verified by synthesis of *N*-desmethylselegiline. Using the *N*-desmethylselegiline standard and selegiline-d_8 as an internal standard, maximum urinary concentrations of *N*-desmethylselegiline (480 ng/ml) occurred at 2 hours after administration of selegiline at 50 mg (PO). These analyses confirmed earlier findings and indicated that *N*-desmethylselegiline is the major urinary metabolite of selegiline following PO administration in horses. Additionally, early detection and rapid elimination characteristics of selegiline metabolites in horse urine suggest that selegiline is rapidly and extensively metabolized in horses. These data indicate that

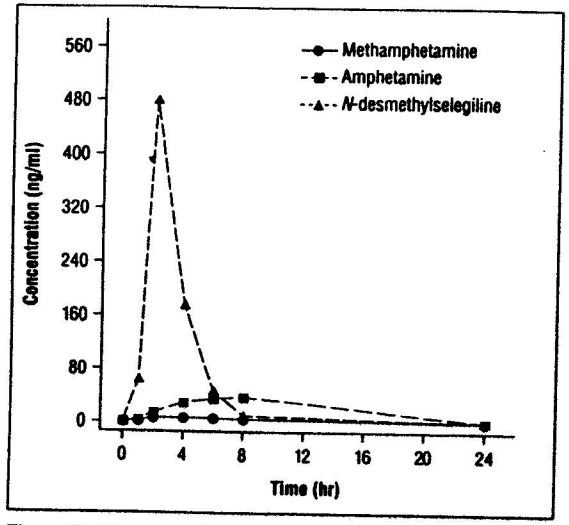

Figure 10. *Time course for selegiline and its equine urinary metabolites from pooled urine samples (n = 4; single PO dose of 50 mg).* N-*desmethylselegiline concentrations were determined using the* N-*desmethylselegiline standard and selegiline-d_8 as internal standard.*

N-desmethylselegiline is most likely the optimal urinary biomarker for detection of recent selegiline administration, and suggest that equine species metabolize selegiline primarily to *N*-desmethylselegiline, which accumulates on formation, and further metabolism to amphetamine is very slow.

In these preliminary behavioral studies, no significant changes in locomotor activities or heart rates were observed following administration of selegiline at 30 mg (IV or PO). Further behavioral studies are planned with higher doses of selegiline.

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Threshold Dose of Three Photosensitizers in Dogs with Spontaneous Tumors

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ABSTRACT

Photodynamic threshold doses in dogs with spontaneous tumors can be achieved through a mathematical model. For this to be clinically relevant, it is important to know the treatment parameters for tissue necrosis. The threshold dose for three photosensitizers (porfimer sodium, aluminum cholorophthalocyanine [AlClPc], and tin ethyl etiopurpurin [SnET2]) commonly used in veterinary chemotherapy protocols was evaluated in 12 dogs with spontaneous tumors. To derive the photodynamic threshold dose, the tissue optical properties of each compound were determined by diffuse reflectance and thus the light fluence rate. Uptake was measured by fluorimetry using tissue solubilization techniques. The threshold values calculated were highest for AlClPc (irradiated 48 hours after administration). The radius of necrosis (4.00 to 5.48 mm) and photosensitizer uptake (3.4 to 6.91 μg/g) were elevated after injection of porfimer sodium. The threshold dose model described here is photosensitizer dependent but independent of photosensitizer uptake and light dose. This study indicates that more photon absorption is needed for tumor necrosis with AlClPc than for either SnET2 or porfimer sodium.

INTRODUCTION

Selectivity of photodynamic therapy (PDT) can be achieved through two mechanisms. The first is by careful placement of the light source and the second by selective localization of photosensitizer drugs (which clear more rapidly from normal tissue than from malignant tumors) within malignant tissues. Ideally, the mathematical models describing light will guide placement of light fiber transport for different tissues. For this study, the intrinsic biologic sensitivity of PDT, which provides information about the requirements for tissue necrosis, was not considered. Knowledge of these parameters is necessary to produce tissue photodamage and improvement in therapeutic conditions but was not necessary for the determination of light requirements for necrosis.

As a model of tissue response to PDT, the photodynamic threshold model has been used by various researchers.[1-4] The model is based on histologic observations of distinct regions of PDT-induced necrosis with clearly defined boundaries compared with areas of tissue that appear normal. Chen and coworkers[1] investigated porfimer sodium in normal rat brain. Patterson and others[2] studied aluminum sul-