CASE REPORT

Thomas C. Kupiec,[1] *Ph.D. and Arvind K. Chaturvedi,*[1] *Ph.D.*

Stereochemical Determination of Selegiline Metabolites in Postmortem Biological Specimens*

REFERENCE: Kupiec TC, Chaturvedi AK. Stereochemical determination of selegiline metabolites in postmortem biological specimens. J Forensic Sci 1999;44(1):222–226.

ABSTRACT: In this study, findings related to an aircraft accident are reported. Biological specimens collected at autopsy from the pilot of the fatal accident and two types of tablets found at the accident scene were submitted for toxicological evaluation. It was determined that the pilot was dead at the crash site and the cause of death was multiple traumatic injuries. The tablets were identified as selegiline and levodopa, commonly prescribed for the treatment of Parkinson's disease. Selegiline, a stereospecific compound, is biotransformed into (−)-*N*-desmethylselegiline, (−)-methamphetamine, and (−)-amphetamine. The latter two *levorotatory* metabolites cannot be easily distinguished by routine analysis from their *dextrorotatory* isomers, which are controlled substances. It was, therefore, prudent to differentiate these isomers to determine if they resulted from the ingestion of a controlled substance, (+)-methamphetamine. Initial immunoassay drug screenings revealed the presence of amphetamine class drugs (867 ng/mL) in urine, amphetamine/methamphetamine (261 ng/mL) in urine, and methamphetamine (46 ng/mL) in blood. The gas chromatography-mass spectrometry (GC/MS) results revealed the presence of methamphetamine in the concentrations of 76 ng/mL of blood and 685 ng/mL of urine. The concentration of amphetamine was 52 ng/mL in blood and 320 ng/mL in urine. To determine the stereospecificity of these amines, the isolated amines from the biosamples were derivatized by a stereospecific agent, (*S*)-(−)-*N*-(trifluoroacetyl)-prolyl chloride, and characterized by a GC/MS method to be *levorotatory*. The 2.14 ratio of (−)-methamphetamine to (−)-amphetamine concentrations in the urine was consistent with a selegiline study in the recent literature. The stereospecific analysis, in conjunction with the history of the pilot being on Parkinson's medications, suggests that the source of these amines was selegiline. This conclusion substantiates the importance of the identification of enantiomers in evaluating and interpreting related analytical results for accident investigations.

[1] Research Chemists, Toxicology and Accident Research Laboratory, Aeromedical Research Division, Civil Aeromedical Institute, Federal Aviation Administration, U.S. Department of Transportation, Oklahoma City, OK.

* This document is disseminated under the sponsorship of the U.S. Department of Transportation in the interest of information exchange. The United States Government assumes no liability for the contents or use thereof.

Presented in part at the 49th Annual Meeting, American Academy of Forensic Sciences, New York, NY, Feb. 1997; it has also been produced as an internal technical report (DOT/FAA/AM-97/14) of the Office of Aviation Medicine, Federal Aviation Administration, U.S. Department of Transportation.

Received 23 March 1998; and in revised form 15 June 1998; accepted 19 June 1998.

KEYWORDS: forensic science, forensic toxicology, selegiline metabolites, methamphetamine, amphetamine, enantiomers, diastereomers, Parkinson's disease, aircraft accident investigation

Selegiline in combination with levodopa is commonly prescribed for the treatment of Parkinson's disease, wherein brain levels of the endogenous dopamine neurotransmitter decrease (1,2). Both medications increase the levels of dopamine: selegiline is a monoamine oxidase inhibitor, while levodopa acts as a precursor of dopamine (1–3). The inhibition of the enzyme prevents the metabolism of endogenous dopamine, thereby increasing its levels in the brain. Selegiline [(*R*-(−)-*N*,α-dimethyl-*N*-(prop-2-ynyl)-phenylethylamine] is rapidly absorbed from the gastrointestinal tract (4). The drug easily crosses the blood-brain barrier and is extensively biotransformed into (−)-*N*-desmethylselegiline, (−)-methamphetamine, and (−)-amphetamine (Fig. 1). Because the chiral center of selegiline is not affected during its metabolism, the three main metabolites remain in the *levorotatory* isomeric [(−)-isomeric] forms (4). *Dextrorotatory* amphetamines—(+)-amphetamine and (+)-methamphetamine—have different pharmacological effects than their respective *levorotatory* isomers and are considered to be controlled substances. It was, therefore, essential to differentiate the *levorotatory* isomers [(−) isomers] from the *dextrorotatory* isomers [(+) isomers].

In the present study, analytical findings related to a general aviation aircraft accident involving one occupant are reported. At the crash site, two types of tablets were found, which were subsequently identified as selegiline and levodopa. Biological samples taken at autopsy from the occupant (pilot) of the fatal accident were submitted for toxicological evaluation. Routine analyses were performed on the submitted samples for a wide variety of drugs, including amphetamine and methamphetamine. These amines were further stereospecifically analyzed and characterized to establish that these amines were *levorotatory*, originating from selegiline. No attempts were made to perform the analysis for selegiline because it is not present in appreciable amounts *in vivo* (4). Analysis of the desmethylselegiline was not pursued as its reference standard was not available. It was also not feasible to conduct analysis for levodopa to distinguish ingested levodopa from the endogenous levodopa.

Case History

A four-seat aircraft (Mooney M20J) with one occupant stalled and impacted the ground near the runway during landing. The

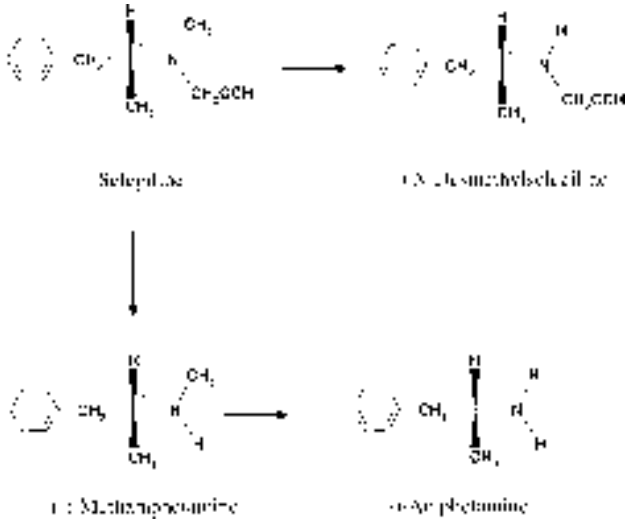

FIG. 1—*Metabolism of selegiline. The chiral centers (asymmetric carbons) are depicted by asterisks.*

accident occurred at 1340 hours on a clear day. At the time of the accident, the meteorological conditions were normal (no cloud, no precipitation, 10-mile (16 km) visibility, and 3-knot wind speed). The aircraft was destroyed, and the 68-year-old Caucasian male pilot (occupant) received fatal injuries. The pilot was dead at the scene. An autopsy was performed on the victim's body. The cadaver measured 1.7 m in length and weighed an estimated 68 kg. It was determined that the cause of death was multiple traumatic injuries, and the manner of death was accidental. The pathological findings were also supportive of the preexisting medical conditions, Parkinson's disease as well as arterialsclerotic heart and vascular disease. The tablets found at the scene were identified as selegiline and levodopa by their shape, color, and markings.

Materials and Methods

Materials

All reagents were of analytical grade and solvents were of high-performance liquid chromatography (HPLC) grade. These chemicals, immunoassay kits, standards, internal standards, and derivatizing agents were obtained from commercial sources. The immunoassay kits for amphetamine/methamphetamine screens were purchased from Abbott Laboratories (Abbott Park, IL) and from Roche Diagnostic Systems (Nutley, NJ). Standards of (+)- and (−)-amphetamines and of (+)- and (−)-methamphetamines were obtained in methanolic solutions from Alltech-Applied Science Labs (State College, PA). The internal standards were supplied as racemic mixtures, (±)-amphetamine-d_8 and (±)-methamphetamine-d_8, by Radian International LLC (Austin, TX). Pentafluoropropionic anhydride (PFPA) was purchased from Pierce (Rockford, IL). The chiral probe used to resolve these enantiomers was (*S*)-(−)-*N*-(trifluoroacetyl)prolyl chloride (TPC). Obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI), this probe was 97% enantiomerically pure and was supplied as 0.1 M solution in dichloromethane.

Screening

Blood and urine samples were tested for common analytes using our laboratory's routine, standard, established procedures. These procedures entailed analyses for ethanol, carboxyhemoglobin, cyanide, and drugs using various chromatographic and spectroscopic techniques, as well as enzymatic and radioactive immunoassays. The urine sample was screened for amphetamine and methamphetamine by the Abbott's ADx® Amphetamine Class and ADx® Amphetamine/Methamphetamine II assays. The blood sample was examined by the Roche's abuscreen® radioimmunoassay to detect methamphetamine.

Quantitative Analysis of Amphetamine and Methamphetamine

Extraction—Four mL of blood and 4 mL of urine were separately spiked with 400 ng of each of the internal standards (±)-amphetamine-d_8 and (±)-methamphetamine-d_8. To the spiked blood sample, 6 mL of acetonitrile was added to precipitate the proteins. Subsequently, the tube was capped, shaken for 5 min, and centrifuged at 120 × *g* for 5 min. The liquid phase was transferred into a new tube and was evaporated to less than 1 mL using a stream of nitrogen; then, 2 mL of 0.1 M phosphate buffer (pH 6.0) was added to the tube. This acetonitrile-mediated precipitation was not necessary for the urine specimen, wherein 2 mL of the 0.1 M phosphate buffer was directly added. The buffered blood extract and urine sample were processed by transferring onto the solid phase extraction columns, following the manufacturer's recommended procedure (Bond Elut Certify™, Varian Sample Preparation Products, Harbor City, CA). The analytes were eluted by 4 mL of 2% ammonium hydroxide in ethyl acetate. Hydrogen chloride was bubbled through the collected eluates to minimize loss of amphetamines.

Derivatization by PFPA—These eluates were evaporated to dryness using a stream of nitrogen, and 50 μL of ethyl acetate and 50 μL of PFPA were added to the residues. The reaction mixtures were incubated for 20 min at 65°C, allowed to cool to ambient temperature, and evaporated to dryness, followed by the addition of 50 μL of ethyl acetate for reconstitution. One μL of the liquid contents was injected onto the gas chromatograph-mass spectrometer (GC/MS).

Derivatization by TPC—To establish the stereospecificity, the amphetamines were extracted from re-accessioned samples as described earlier and derivatized by using 50 μL of the TPC chiral probe solution (5,6). Following the 20-min incubation at 65°C, the evaporation to dryness, and the reconstitution in 50 μL of ethyl acetate, 1 μL of the contents was injected onto the GC/MS system. The chemical reaction for the derivatization is depicted in Fig. 2.

Instrumentation

A Hewlett Packard gas chromatograph (Model 5890)-mass spectrometer (MS Engine; Model 5989) system was used during the analysis. A crosslinked 5% phenyl methyl silicone column (15 m × 0.25-mm inside diameter; 0.25-μm film thickness) was used. Helium was the carrier gas with a flow of 1 mL/min. The injection volume was 1 μL in the splitless mode, with a purge time of 0.5 min. The injector temperature was maintained at 200°C. The transfer line was set at 275°C. The acquisition was in electron impact mode of ionization using selective ion monitoring. For the PFPA derivatives, the oven temperature was kept at 90°C for 1 min. It was then increased to 150°C at 8°C/min and then to 280°C at 40°C/min; the final temperature of 280°C was maintained for 2 min. For the TPC derivatives, the oven temperature was set at 90°C

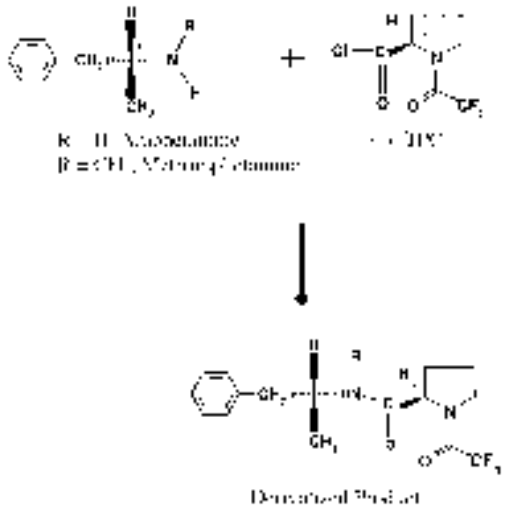

FIG. 2—*Derivatization of amphetamine and methamphetamine by (S)-(−)-N-(trifluoroacetyl)prolyl chloride (TPC). The asterisks indicate chiral centers (asymmetric carbons) in the molecules.*

TABLE 1—*Screening findings.*

Analyte	Specimen	Concentration, ng/mL
Amphetamine class drugs	Urine	867
Amphetamine/methamphetamine	Urine	261
Acetaminophen	Urine	4800
Methamphetamine	Blood	46

for 1 min, increasing to 240°C at a rate of 10°C/min; the temperature was then increased to 285°C at a rate of 40°C/min and kept for 2 min.

Calibration Curves

Separate calibration curves for amphetamine and methamphetamine in blood, as well as in urine, were constructed. The concentration range of each of these amines for obtaining the curves was 10.24–1000 ng/mL for blood, while it was 31.25–1000 ng/mL for urine. The regression analysis disclosed that the curves were linear with the correlation coefficients ranging from 0.9993 to 0.9998.

Results

Screening

As given in Table 1, routine toxicological evaluation of the urine disclosed the presence of amphetamine class drugs at 867 ng/mL and amphetamine/methamphetamine at 261 ng/mL. The former value was obtained from the Abbott's ADx® Amphetamine Class assay, while the latter value was from the Abbott's ADx® Amphetamine/Methamphetamine II assay. The screening of the blood by the Roche's abuscreen® assay disclosed the presence of 46 ng/mL methamphetamine. In addition, acetaminophen was found at a level of 4800 ng/mL in the urine by the ADx® assay (Abbott Laboratories, Abbott Park, IL). Ethanol, combustion gases, or other common drugs were not detected in the urine and blood.

Confirmation and Quantitation

The PFPA derivatization method disclosed 320 ng/mL amphetamine and 685 ng/mL methamphetamine in the urine and 52 ng/mL amphetamine and 76 ng/mL methamphetamine in the blood (Table 2). Under our GC/MS conditions, both amphetamine and methamphetamine clearly separated from each other. The retention times were 4.82 min for amphetamine and 6.23 min for methamphetamine. The respective deuterated internal standards eluted approximately 0.08 min prior to the corresponding nondeuterated amines. All these amines were characterized by the monitoring of selective ions. Although the internal standards were in racemic mixture forms, no separation of the (−) isomers from the (+) isomers was noted; only one peak was noticed with either internal standard. There were no interfering peaks.

TABLE 2—*Confirmation and quantitation of amphetamine and methamphetamine following derivatization with PFPA by the GC/MS method.*

Analyte	Specimen	Concentration, ng/mL
Amphetamine	Urine	320
Methamphetamine	Urine	685
Amphetamine	Blood	52
Methamphetamine	Blood	76

TABLE 3—*GC/MS analytical characteristics used in the differentiation of diastereomers of (−) and (+) amphetamine and of (−) and (+) methamphetamine standards utilizing the stereospecific derivatizing agent (S)-(−)-N-(trifluoroacetyl)prolyl chloride (TPC).*

Stereospecific Amine	Retention Time, min	Molecular Ion, m/z
(−)-Amphetamine-d_8	11.99	240
(−)-Amphetamine	12.05	237
(+)-Amphetamine-d_8	12.24	240
(+)-Amphetamine	12.31	237
(−)-Methamphetamine-d_8	13.35	258
(−)-Methamphetamine	13.47	251
(+)-Methamphetamine-d_8	13.64	258
(+)-Methamphetamine	13.71	251

Stereochemical Differentiation

Analyses of the TPC derivatized products clearly suggested that the amphetamines found in the blood and urine were *levorotatory*. Under the instrumental conditions, the (−) isomers distinctly separated from their respective (+) isomers. With the submitted samples, primary peaks corresponding to the (−) isomers of amphetamine and of methamphetamine were evident. Upon the TPC derivatization, the racemic mixture of deuterated analogs of either amine eluted approximately 0.3 min apart. Both amines were confirmed and characterized by their retention times and characteristic ions by utilizing the GC/MS system. The retention times, along with molecular ions, of the different diastereomers of these amines and of the deuterated internal standards (racemic mixtures) are presented in Table 3. As is evident from Fig. 3, there was a baseline chromatographic separation of the diastereomers of the internal standards, as well as of amphetamines and methamphetamines in the processed urine sample. However, a relatively small peak was noted at 13.60 min after (+)-methamphetamine-d_8; the area of the small peak was 3.9% of the (−)-methamphetamine peak. A similar peak of the same proportion was also noted with the blood sample. Such a peak was not detected with the deuterated

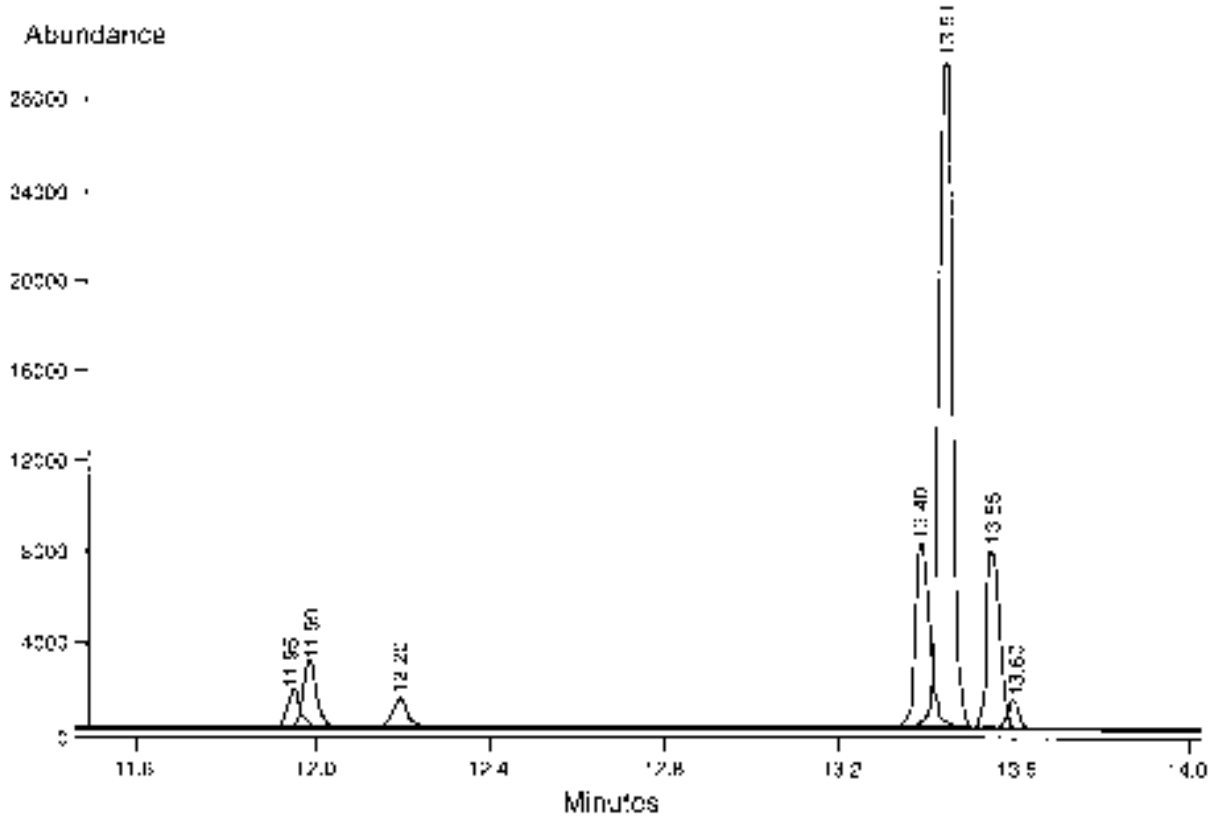

FIG. 3—*A chromatographic separation of TPC-derivatized diastereomers of (−)-amphetamine and (−)-methamphetamine. Amphetamines were isolated from the urine sample. Deuterated racemic mixtures of amphetamine and of methamphetamine were used as internal standards. Details are described in the materials and methods section. In the chromatogram, the analytes' retention times (min) were: (−)-amphetamine-*d_8 *(11.95), (−)-amphetamine (11.99), (+)-amphetamine-*d_8 *(12.20), (−)-methamphetamine-*d_8 *(13.40), (−)-methamphetamine (13.51), and (+)-methamphetamine-*d_8 *(13.55).*

and nondeuterated TPC-derivatized standards. The observed peak could be attributed to an undetermined substance present in the biological matrixes.

Discussion

It is imperative to detect, confirm, and quantitate drugs in biological samples to facilitate aircraft accident investigations. Such findings indicate whether the victim was taking any prescribed/nonprescribed medications or illicit drugs, and whether the victim was in compliance with the physician's orders by taking or not taking the medication(s). In those medications where stereospecificity plays an important role in exhibiting pharmacological effects, there is also a need to analytically evaluate whether the compound is *levorotatory* or *dextrorotatory*. Such evaluation is important because only a particular isomer may produce a selective biological response, or a particular isomer may fall under a specific category of the controlled substances. Therefore, the stereospecificity of the analyte should be clearly established for medicolegal purposes.

The toxicological evaluation of the submitted biological samples revealed the presence of *levorotatory* isomers of amphetamine and methamphetamine. Their source could have been selegiline, as this drug biotransforms into these amines. This possibility is supported by the identification of the tablets found at the scene as selegiline and levodopa. Furthermore, the pilot had a medical history of Parkinson's disease and was being treated for this disorder. Since selegiline is completely metabolized into the three major metabolites, the parent compound is not present in appreciable amounts in the biological system (4). Although a GC/MS-based qualitative identification of (−)-*N*-desmethylselegiline would have been even useful in directly establishing the origin of these amphetamines, the chiral derivatizing agent was instrumental in demonstrating the presence of their *levorotatory* isomers in the biosamples. The used analytical procedure was able to differentiate *levorotatory* isomers from their *dextrorotatory* isomers. The absence of *dextrorotatory* amphetamine and methamphetamine clearly indicated that the victim did not consume the controlled form of these substances. If the differentiation between the stereoisomers of amphetamine and methamphetamine had not been made, the individual might have been falsely accused of using the controlled substances.

The concentration ratios of methamphetamine to amphetamine in urine have been used to establish the origin of these amines (6,7). According to Romberg et al. (6), a ratio of about 2.80 for (−)-methamphetamine to (−)-amphetamine concentrations in the urine of selegiline users has been observed. This ratio is close to the 2.14 ratio calculated from the present study, further suggesting the origin of these amines to be selegiline. (−)-Methamphetamine and (−)-amphetamine could also be present in the urine of the users of Vicks Inhaler™ (8), but the ratio in such cases usually exceeds 8 (6), thereby ruling out the inhaler as the source of these amines in this case.

In the present study, the concentrations of amphetamine and methamphetamine were derived from the PFPA derivatization method, which does not differentiate the *levorotatory* isomers from the respective *dextrorotatory* isomers. In this method, both (−)

and (+) isomers elute at the same time. The quantitative values represented the total amounts of both optical isomers. To establish the correctness of the quantitative results, known concentrations of (−)-amphetamine and (−)-methamphetamine were quantitatively analyzed, using the PFPA method and calibration curve. The experimental values for these amines were very close to the expected concentrations. This observation suggests that the PFPA method is suitable for the quantitation of amines and is independent of their optical property, but the TPC derivatization method should be used to distinguish the *levorotatory* isomers from the respective *dextrorotatory* isomers. This suggestion is further supported by the fact that only one peak was observed when the deuterated internal standard (a racemic mixture) of either amine was derivatized with PFPA, whereas the racemic mixture derivatized with TPC produced two distinct peaks. These peaks were characterized by the retention times of the respective (−) and (+) isomers of amphetamine or methamphetamine.

Conclusion

This study suggests that the source of amphetamine and methamphetamine in the samples was selegiline. This conclusion is based on the stereospecific analysis of these amines in conjunction with the clinical history of the pilot and the presence of two tablets identified as selegiline and levodopa. With optically active medications, the stereospecificity determination is essential for the correct interpretation of the toxicological findings to facilitate accident investigations.

References

1. Cedarbaum JM, Schleifer LS. Drugs for Parkinson's disease, spasticity, and acute muscle spasms. In: Gilman AG, Rall TW, Nies AS, Taylor P, editors. Goodman and Gilman's the pharmacological basis of therapeutics, 8th ed. New York, NY: Pergamon Press, 1990;463–84.
2. Cooper JR, Bloom FE, Roth RH. The biochemical basis of neuropharmacology, 6th ed. New York, NY: Oxford University Press, 1991;285–337.
3. USP DI®, Drug information for the health care professional, United States Pharmacopeial Convention, Inc., 11th ed., Vol. 1B. Kingsport, TN: Arcata Graphics, 1991;1665 & 2309.
4. Meeker JE, Reynolds PC. Postmortem tissue methamphetamine concentrations following selegiline administration. J Anal Toxicol 1990;14:330–1.
5. Fitzgerald RL, Ramos JM Jr, Bogema SC, Poklis A. Resolution of methamphetamine stereoisomers in urine drug testing: urinary excretion of *R*(−)-methamphetamine following use of nasal inhalers. J Anal Toxicol 1988;12:255–9.
6. Romberg RW, Needleman SB, Snyder JJ, Greedan A. Methamphetamine and amphetamine derived from the metabolism of selegiline. J Forensic Sci 1995;40:1100–2.
7. Kikura R, Ishigami A, Nakahara Y. Studies on comparison of metabolites in urine between deprenyl and methamphetamine. III. Enantiomeric composition analysis of metabolites in mouse urine by HPLC with GITC chiral reagent. Japanese J Toxicol Env Health 1992;38:136–41.
8. Cody JT, Schwarzhoff R. Interpretation of methamphetamine and amphetamine enantiomer data. J Anal Toxicol 1993;17:321–6.

Additional information and reprint requests:
Dr. Arvind K. Chaturvedi
Toxicology and Accident Research Laboratory (AAM-610)
FAA Civil Aeromedical Institute
P.O. Box 25082
Oklahoma City, OK 73125–5066.