Enantiomeric Determination of Amphetamine and Methamphetamine in Urine by Precolumn Derivatization with Marfey's Reagent and HPLC

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Abstract

An analytical method was developed for enantiomeric determination of amphetamine and methamphetamine in human urine. The enantiomers were isolated from urine by solid-phase extraction, and diastereomers were formed by derivatization with the chiral Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-1-aniline amide). The diastereomers were separated by reversed-phase high-performance liquid chromatography in a water/methanol mobile phase and detected by absorbance spectrophotometry at 340 nm. Linear standard curves were obtained for all four enantiomers over a concentration range of 0.16–1.00 mg/L in urine. The detection limit was 0.16 mg/L urine for each enantiomer, and the limit of quantitation was 0.40 mg/L. The urine of 10 decedents was analyzed by this method and by a previously published precolumn derivatization procedure using (-)-L-(9-fluorenyl)ethyl chloroformate (FLEC) as the derivatizing agent and fluorescence detection. Comparison of the results of the two methods by linear regression showed comparable results for both d-amphetamine and d-methamphetamine. Neither method detected the presence of the L-enantiomers in the urine samples.

Introduction

A variety of procedures have been reported for the enantiomeric determination of amphetamine (AMP) and methamphetamine (METH) in human urine (1–3). Most procedures involve liquid-liquid extraction (LLE) (2–4) or solid-phase extraction (SPE) (1,5) of the enantiomers from urine before analysis. Varesio and Veuthey (6) suggest that LLE is more rapid but that SPE methods give a cleaner extract. Another procedure involves a simple filtration of urine before analysis (7).

Enantiomeric determinations typically involve some form of gas or liquid chromatographic (GC, LC) separation. Precolumn derivatization with a chiral reagent to form diastereomers is a common technique that allows the use of achiral chromatographic analytical columns for the separation (4,7–9). Achiral columns are commonly available and less expensive than chiral stationary phase columns. Chiral stationary phase methods, particularly with cycloextrinsics, have also been reported for the enantiomeric determinations of AMP and METH (6,10,11). Detection methods include typical GC detectors, mass spectrometry (GC-MS), UV-vis absorption spectrophotometry, and fluorescence emission. Capillary zone electrophoresis with a variety of cycloextrinsics and chiral cellulosics as chiral selectors is also receiving attention (6,11).

This work reports an SPE of AMP and METH from human urine followed by a precolumn derivatization of the enantiomers with the chiral Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-aniline amide) (12) and subsequent high-performance liquid chromatographic (HPLC) separation on a C-18 reversed-phase achiral column and spectrophotometric detection. Authentic urine samples from decedents were analyzed by this reported Marfey's reagent method and also by an LLE procedure followed by a precolumn derivatization with (-)-fluorenylethyl chloroformate [(-)-FLEC] (4).

Experimental

Materials

Racemic METH hydrochloride and AMP sulfate were obtained from Sigma Chemical (St. Louis, MO). HPLC-grade methanol, reagent-grade acetic acid, and ammonium hydroxide were procured from EM Science (Gibbstown, NJ). Baker-analyzed-grade sodium bicarbonate and monobasic and dibasic sodium phosphates were obtained from J.T. Baker (Phillipsburg, NJ). Benzylamine hydrochloride, methylene chloride, and isopropyl alcohol were acquired from Aldrich Chemical (Milwaukee, WI). ACS-certified N,N-dimethylformamide was obtained from Fisher Scientific (Fair Lawn, NJ). The derivatizing agent, Marfey's reagent (FDAA), was obtained from Pierce Chemical (Rockford, IL).
Water was purified using a Barnstead (Dubuque, IA) ion-exchange NANOpure Ultra Pure water system (model D4741) with a 2-μm final filter.

**Instrumentation and apparatus**

The chromatographic system consisted of a Waters Associates (Milford, MA) model 590 programmable solvent delivery pump and a model 481 variable-wavelength detector LC spectrophotometer operated at 340 nm, a Rheodyne sample injector model 7125 (Cotati, CA) with a 20-μL sample loop, a Gateway 2000 computer, and Chrom Perfect Direct 4I software (version 5.07) from Justice Innovations (Palo Alto, CA). The HPLC reversed-phase analytical column (Adsorbosphere HS, C-18, 150 mm x 4.6-mm i.d., 5-μm particle size) was from Alltech Associates (Deerfield, IL) and was fitted with a 7.5-mm x 4.6-mm guard column with the same packing as the analytical column.

Solid-phase extractions were accomplished using World Wide Monitoring bonded-phase extraction sorbents (Clean Screen F1CZ20, 200 mg/10 mL, 8.5-ram bed diameter, United Chemical Technologies, Bristol, PA), and a 12-port Visiprep disposable (DL) SPE vacuum manifold from Supelco (model 5-7044, Bellefonte, PA).

Two- and four-millilitter reacti-vials from Pierce were used for the collection of solid-phase eluants and in the derivatization reactions. A Pierce Reacti-Therm heating/stirring module (series 344) was used for heating the derivatizing reactions, and a Pierce model 1780 Reacti-vap evaporating unit was used to evaporate solid-phase eluants.

Calibrated, fixed-volume Eppendorf micropipet syringes (1–1000 μL, model 3130) from Brinkmann Instruments (Westbury, NY) were used for the precise measurements of microliter volumes of solutions.

**Reagents and solutions**

Solutions of 1.0M acetic acid, 0.10M phosphate buffer (pH 6.00), 1.0M hydrochloric acid, 1.0M sodium bicarbonate, and 0.1%(w/v) Marfey's reagent in HPLC-grade acetone were prepared as needed. The SPE elution solvent, methylene chloride (MeCl2)/isopropyl alcohol (IPA)/aqueous ammonia (NH3) (78:20:2, v/v), was prepared daily.

Stock solutions of racemic AMP and METH at 1.0 mg/L were each prepared in HPLC-grade methanol. All concentrations reported here are expressed the free base. A separate stock solution of the internal standard, benzylamine, was prepared in methanol at 500 mg/L.

**Methods**

**Solid-phase extraction**

Two milliliters of 0.10M phosphate buffer was added to 5.00 mL of each urine sample and standard and then vortex mixed. An SPE column was conditioned with 3 mL of the MeCl2/IPA/NH3 solution followed by 3 mL of methanol, 3 mL of water, and 1 mL of phosphate buffer, all without the application of any vacuum. The 5-mL urine sample was added and drawn through the SPE column at 1–2 mL/min with a vacuum of 1–2 in. of mercury. The column was washed with 3 mL of water, 1 mL of 1.0M acetic acid, and 3 mL of methanol. Air was drawn through the column with vacuum at 10 in. or more of mercury for 5 min. The AMP and METH enantiomers were eluted with 3 mL of MeCl2/IPA/NH3 solution and collected in 5-mL reacti-vials with no applied vacuum (13).

**Derivatization**

Reacti-vials with the eluted samples were placed in a heating block of the Pierce Reacti-Therm unit fitted with a Reacti-Vap accessory, and 30 μL of dimethyl formamide was added.
The solution was evaporated with a stream of nitrogen at less than 40°C, and 100 μL of water was added, followed by 20 μL of 1M NaHCO₃. The vials were vortex mixed, and 100 μL of Marfey’s reagent was added. The sample was vortex mixed for 2–3 min and placed in the heating block of a Reacti-Therm unit at 40–45°C for 1 h. The mixture was cooled to room temperature, and 40 μL of 1M HCl was added. The vial with the reaction mixture was placed in the heating block of the Reacti-Therm unit and evaporated to dryness at less than 40°C under a stream of nitrogen.

**Standard curves**

Solutions for standard curves were prepared by spiking drug-free urine with the stock methanol solutions of AMP, METH, and benzylamine as an internal standard. Concentrations of each AMP and METH enantiomer were 0.10–1.00 mg/L with a benzylamine concentration of 0.40 mg/L. Six-point standard curves were obtained by carrying these solutions through the entire SPE, derivatization, and chromatographic procedure.

**Chromatography**

After evaporation, the residue was reconstituted in 500 μL of mobile phase, methanol/water (60:40), vortex mixed for approximately 2 min, and injected onto the chromatographic column. The mobile phase was degassed with helium for 15 min. Separations were performed isocratically at ambient temperature with a mobile-phase flow rate of 1.0 mL/min. Data were acquired for a 45-min period. The analytical column was flushed with 100% methanol between each injection and at the end of the day.

**Analysis of authentic urine samples**

Samples of urine from decedents were provided through the courtesy of the Maricopa County medical examiner’s office. The samples were diluted 10-fold with phosphate buffer, and benzylamine, 0.40 mg/L, was added. The solutions were then extracted, derivatized, and chromatographed.

**Results and Discussion**

**Derivatization**

Enantiomerically pure Marfey’s reagent was first used as a derivatizing agent to form diastereomers of amino acids: their subsequent HPLC separation and determination (12). Marfey’s original derivatization procedure was used with only the modification of a 0.1% (w/v) solution of the reagent in acetone instead of a 1% (w/v) solution. This reduced the size of the early HPLC peaks, which yielded better resolution of the benzylamine internal standard peak. The conditions of pH, temperature, and time of reaction were the same as in Marfey’s original work. Calmes et al. (14) reported its use to determine the chiral purity of benzylic amines. Rizzi and co-workers (10,11) compared the separations achieved with Marfey’s derivatives of AMP, METH, and various analogues to those obtained with cyclodextrin-based HPLC and capillary zone electrophoresis separations. Their work was not extended to an application of the determination of the substances in human urine.

Reactions were carried out at 40°C for 1 and 4.75 h with no change in the ratio of the l/d diastereomer peak heights of either METH or AMP. There is, therefore, no appreciable racemization under these conditions.

**Chromatograms**

Figure 1 is a chromatogram of a drug-free urine sample spiked with 0.80 mg/L each of METH and AMP enantiomers that underwent the experimental procedure. Elution times and capacity factors for the internal standard and various diastereomers were benzylamine, 15.1 min (k’ = 7.8); l-METH, 22.7 min (k’ = 12.4); d-METH, 24.9 min (k’ = 13.7); l-AMP, 32.0 min (k’ = 17.8); and d-AMP, 35.7 min (k’ = 20.0). The resolutions (Rs) between the METH and AMP peaks were 1.86 and 2.25, respectively, with baseline resolution between the sets of METH and AMP diastereomers. The order of elution of sets of diastereomers is consistent with the order of elution of amino acid diastereomers on reversed-phase columns (i.e., l-diastereomers-l-Marfey’s are eluted before d-diastereomers-l-Marfey’s) (12).

**Standard curves**

Standard curves of the ratio of peak heights of the diastereomer analyte/benzylamine internal standard versus the concentration of the analyte in urine were obtained for each enantiomer with six standards in the range of 0.10–1.00 mg/L. Chrom Perfect software yielded linear regressions with inverse-squared weighting of the data points. Nine typical curves were linear with coefficients of determination from 0.972 to 0.990. The Chrom Perfect software also returned the average absolute percent error of the data points relative to the regression curve, which varied from 5.0 to 6.1% for the nine standard curves obtained on different days. The linearity of all the curves suggested that analysis of samples could be accomplished with satisfactory precision with the inclusion of a high and a low standard in the runs of the samples, rather than six standards. The limit of
Marfey's reagent method. The regression plots yielded coefficients of determination of 0.991 (d-METH) and 0.988 (d-AMP).

Conclusion

Marfey's reagent was shown to be a viable chiral derivatizing agent for AMP and METH. Combined with an SPE cleanup of human urine, it can be used for enantiomeric determinations with a limits of detection and quantitation of 0.16 and 0.40 mg/L respectively for each enantiomer. The SPE-Marfey’s procedure gave results comparable with those obtained from the more sensitive LLE and derivatization with FLEC and fluorometric detection. The procedure of choice for the enantiomeric determinations of these analytes largely depends on the equipment found in a given laboratory, the previous experience of the staff, and the desired limits of quantitation.

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References


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