



Accurate Biological Testing for Amphetamine and Methamphetamine Abuse Using Chiral HPLC and MS Detection

In this study, optimized methods are presented for sample preparation and chiral chromatography for the LC/MS analysis of amphetamine and methamphetamine enantiomers in urine.

Introduction

Methamphetamine and amphetamine, powerful CNS stimulants that have a variety of ethical uses, have side effects of increased confidence, sociability, and energy that have resulted in their extensive abuse as recreational psychoactive drugs. This extends to their abuse in sports because of the additional properties of increased mental alertness and suppression of fatigue.¹ Negative side effects include hypertension and tachycardia. In the usually uncontrolled situations of drug abuse, they exhibit similar psychoactive effects, although amphetamine is generally less potent than methamphetamine.

Illicit use of these drugs continues to be high. Public Health England recently reported that the number of people stating their main injecting drug to be amphetamines and amphetamine-type substances (such as mephedrone) nearly tripled between 2002 and 2012.² Additionally, unlike the less-volatile amphetamine sulphate, methamphetamine hydrochloride (sometimes known as “ice” or “crystal meth”) can be smoked, giving highs that are similar to crack cocaine, although significantly longer lasting. It has been reported that routine co-monitoring of methamphetamine and amphetamine is not normally carried out, so there is little information on which is more widely used. It was noted however, that amphetamine is more widely available than methamphetamine in Europe, whereas the reverse is true for North America and Asia.³

Chirality

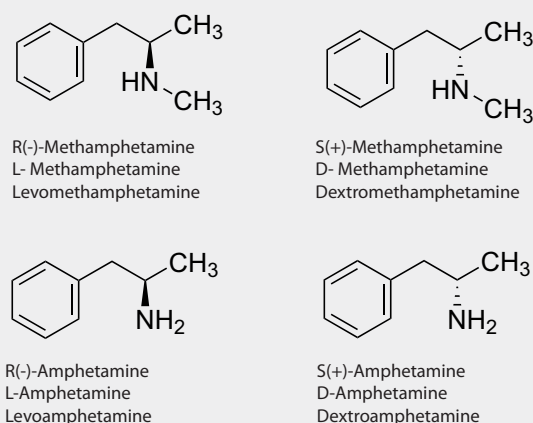
Amphetamine and methamphetamine are chiral molecules (Figure 1). In both cases the D-enantiomer has greater biological activity than the L-enantiomer. Although methamphetamine is a controlled substance (Class A in Europe and Schedule II in the US, both as individual enantiomers and as the racemate), the L-isomer is in fact used in several over-the-counter medicines (e.g. Vicks® inhaler in North America) and so can alter the true level arising from abuse of the drug. The analysis is further complicated by the fact that L-methamphetamine is also a metabolite of certain therapeutic drugs, such as selegiline, a treatment for early-onset Parkinson's disease, depression, and dementia. Amphetamine quantification is more complex, again, simply because it is also the main metabolite of methamphetamine. The traditionally used method for drug analysis, immunoassay, cannot distinguish between the enantiomers and so can easily give incomplete, inconclusive results.

When methamphetamine is sold illegally, it is usually as the D-enantiomer or as the racemate. When ingested, a dose may vary

from several tens to several hundreds of milligrams, depending on the purity and the isomeric composition, and will metabolize to amphetamine and 4-hydroxymethamphetamine.³ Amphetamine metabolizes mainly to 1-phenyl-2-propanone, with smaller amounts of 4-hydroxyamphetamine. However, since up to 54% of methamphetamine is excreted unchanged and 10-23% as amphetamine following oral ingestion, it is usually the parent drug that is monitored.¹

Culpability for the illicit use of amphetamine and methamphetamine rests on the ability to distinguish the contribution to the measured abused product from possible alternative sources of the L-enantiomer. Therefore, the separation of the enantiomers is a more accurate approach for testing. A chiral method can also be used to indicate the synthetic route that was used by the illegal source, which can be useful as part of a criminal investigation. Indeed, detection of just the D-methamphetamine isomer has been found in product prepared through either ephedrine or pseudoephedrine.⁴

Figure 1. Structures of D- and L-Amphetamine and Methamphetamine



(continued on next page)



Analytical Method Development

Chiral GC and LC are both available as possible bioanalytical methods, although LC/MS/MS is becoming the method of choice for its greater flexibility and coverage of a far wider range of compounds of toxicological interest.

GC inevitably involves derivatization for these types of molecules, usually as the trifluoroacetate on chiral GC phases. An alternative in a recent study utilized (R)-(-)- α -methoxy- α -(trifluoromethyl) phenylacetyl chloride (MTPA), separating the enantiomers on an achiral polysiloxane phase.⁴ It provided a method that was suitable for samples also containing the related MDA, MDMA, and MDEA, with a limit of quantification (LOQ) in urine of <10 μ g/L. In general, however, sample preparation and methodology for HPLC is simpler and more amenable to biological samples and so was chosen for this study. A method was developed and optimized for resolution, retention, and LOQ.

HPLC Method Development

Astec® CHIROBIOTIC® HPLC chiral stationary phases (CSPs), based on bonded macrocyclic glycopeptides, were chosen because of their high compatibility with mass spectrometric (MS) detection and their suitability for polar molecules. Another advantage of Astec CHIROBIOTIC CSPs for clinical and forensic applications is their robustness and ability to withstand repeated injections of biological samples under conditions that other CSPs cannot tolerate. Astec CHIROBIOTIC CSPs show enantioselectivity in both reversed-phase and polar ionic mobile phases, the latter defined as mobile phases containing >90% organic modifier with water and ionic additives comprising <10% in varying proportions.

Methamphetamine was run through the reversed-phase and polar ionic mode chiral screens and found to show the highest selectivity on Astec CHIROBIOTIC V2 in the polar ionic mode with a choice of either acetic acid/ammonium hydroxide or ammonium trifluoroacetate salt additives. Both of these additive systems were investigated as part of the optimization process.

Although typically the choice for the polar ionic mode is 100% organic, essentially a non-aqueous phase, up to 10% water can be added before the mechanism changes to a reversed-phase one. The additives used in the polar ionic mode are typically an acid and a base or a volatile salt. Their function is to enhance ionic interactions with the ionizable surface of the CSP, and have a concomitant beneficial effect of increasing analyte ionization in the MS. The addition of water to the mobile phase, up to 10%, maintains the solubility of the ionic additives and generally improves the robustness and repeatability of the method.

The Astec CHIROBIOTIC separation mechanism, as for all chiral stationary phases, is likely a complex one. The surface of the macrocyclic glycopeptides includes a variety of possible interactive sites, such as amide (peptide), phenolic, ionic (amine or carboxylic acid) and aromatic chloro moieties, in addition to inclusion complexing pockets. Astec CHIROBIOTIC V2, which uses vancomycin as the chiral selector, has a carboxylic acid group and is therefore particularly suited to the separation of many amines, while the other functional groups are thought to enhance the separation through hydrogen bonding.

Experimental Separation of Methamphetamine Enantiomers

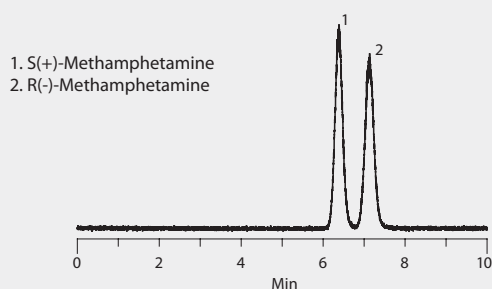
Using a 15 cm x 4.6 mm, 5 μ m Astec CHIROBIOTIC V2 column, two polar ionic mobile phases were compared for retention and resolution. Both comprised methanol:water (95:5). The first mobile phase used acetic acid and ammonium hydroxide (0.1:0.02) as the ionic additives. The second used 0.05% ammonium trifluoroacetate. Results appear in **Figure 2**. In both cases, baseline resolution was obtained, with the D- or S(+) enantiomer eluting before the L- or R(-) enantiomer. Ammonium trifluoroacetate gave less retention and resolution.

These two mobile phase systems were then compared for sensitivity. With data shown in **Figure 3**, by comparing the signal to noise ratios, it was determined that the method employing the acetic acid/ammonium hydroxide mobile phase provided the lowest LOQ of less than 12.5 ng/mL and so was chosen as the optimum system. A calibration curve was also created and the method was found to be linear from 12.5 to 240 ng/mL for R(-)-methamphetamine. The same chromatographic conditions were tested for amphetamine and found to provide high resolution, with slightly lower retention than methamphetamine (**Figure 4**).

Figure 2. Chiral LC/MS Analysis of Methamphetamine Enantiomers on Astec CHIROBIOTIC V2 in Different Mobile Phase Additive Systems

column: Astec CHIROBIOTIC V2, 15 cm x 4.6 mm, 5 μ m particles (15023AST)
mobile phase 1: [A] methanol; [B] water; [C] acetic acid; [D] ammonium hydroxide; (95:5:0.1:0.02, A:B:C:D)
mobile phase 2: methanol:water (95:5) containing 0.05% ammonium trifluoroacetate
flow rate: 1.0 mL/min
pressure: mobile phase 1, 1257 psi (87 bar); mobile phase 2, 1220 psi (84 bar)
column temp.: 20 °C
detector: MS, SIR 150.1, 2 Hz (Waters ACQUITY QDa)
injection: 2 μ L
sample: (+/-)-Methamphetamine, 500 ng/mL in methanol

Mobile Phase 1: Ammonium acetate in methanol:water



Mobile Phase 2: Ammonium trifluoroacetate in methanol:water

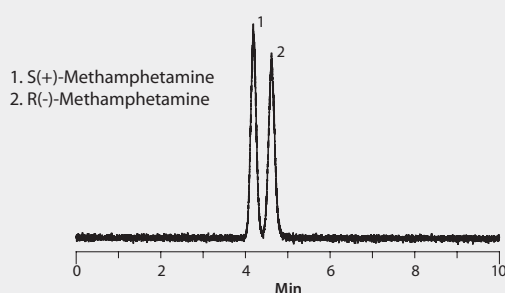
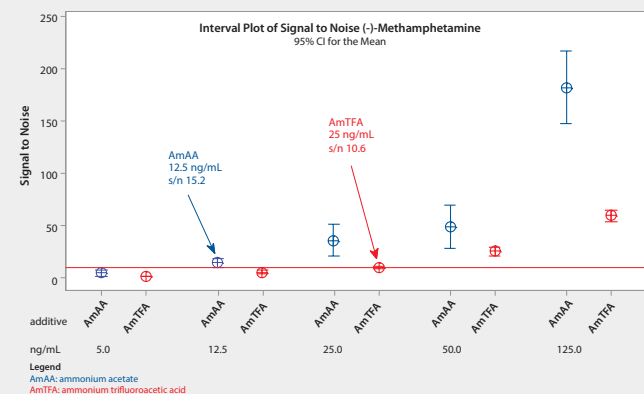


Figure 3. A Study of the S/N Ratio for Optimization of LOQ



Conditions in Figure 2.

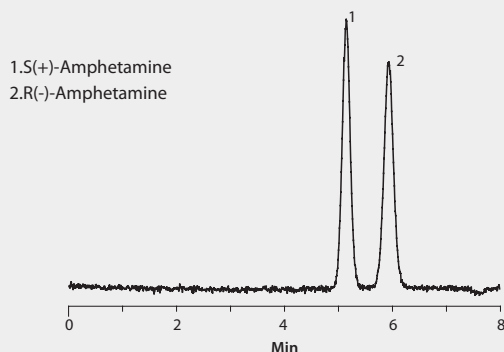
Figure 4. Chiral LC/MS Analysis of Amphetamine Enantiomers on Astec CHIROBIOTIC V2

Conditions Same as Figure 2 Except:

mobile phase: [A] methanol; [B] water; [C] acetic acid; [D] ammonium hydroxide;
(95:5:0.1:0.02, A:B:C:D)

pressure: 1257 psi (87 bar)

sample: 50 ng/mL each enantiomer in methanol



Simultaneous Analysis of Amphetamine and Methamphetamine

Although the compounds are not isobaric, a simultaneous method was sought that would provide baseline resolution of all four enantiomers. The optimal conditions for methamphetamine and amphetamine analyzed separately resulted in a coelution of two of the four peaks for the simultaneous separation of all four enantiomers. While MS takes sufficient care of this overlap, a study was also undertaken to optimize the method. Changes in the acid/base ratio were studied for their impact on resolution and a change to an Astec CHIROBIOTIC V column (same chiral selector but different bonding chemistry) was investigated. However, neither approach provided the required resolution. Success was achieved by simply increasing the column length and temperature (Figure 5).

Importance of Sample Preparation

Effective sample prep removes sample matrix components that reduce sensitivity, decrease the method's robustness, or both. A comparison was carried out between liquid-liquid extraction and SPE for the optimum extraction of methamphetamine from urine.

Figure 5. Simultaneous LC/MS Analysis of the Enantiomers of Amphetamine and Methamphetamine on Astec CHIROBIOTIC V2

column: Astec CHIROBIOTIC V2, 25 cm x 4.6 mm, 5 μ m particles (15024AST)
mobile phase: [A] methanol; [B] water; [C] acetic acid; [D] ammonium hydroxide;
(95:5:0.1:0.02, A:B:C:D)

flow rate: 1.0 mL/min

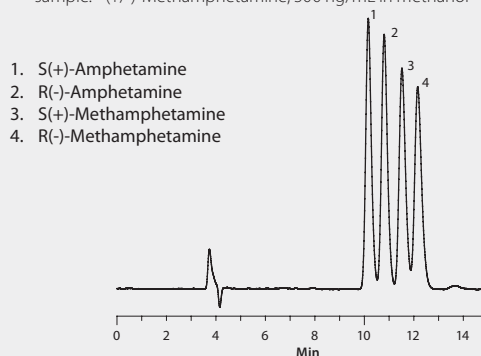
pressure: 840 psi (58 bar)

column temp.: 40 °C

detector: UV, 205 nm

injection: 2 μ L

sample: (+/-)-Methamphetamine, 500 ng/mL in methanol



Liquid-Liquid Extraction

Following a previously published method, a 125 μ L aliquot of synthetic urine was spiked with methamphetamine (125 ng/mL each enantiomer) and 1 mL diethyl ether was added. After vortexing for 30 minutes, the sample was centrifuged at 10,000 rpm for 10 minutes. A 500 μ L aliquot of the upper layer (organic) was taken and evaporated to dryness under nitrogen at 55 °C. This was then reconstituted in 500 μ L mobile phase and filtered through a 0.22 μ m PVDF filter.⁶

Solid Phase Extraction

For the SPE method, 1 mL of synthetic urine was spiked with 25 ng/mL of each enantiomer, and acidified to pH 3-4 with formic acid. After conditioning a Supel™-Select SCX SPE 96-well plate (30 mg/well) with 1 mL 1% formic acid in acetonitrile followed by 1 mL water, it was loaded with 1 mL of the urine sample. The plate was washed with 2 mL water followed by 1 mL 25% methanol. For amphetamine, 1 mL of 5 mM dibasic ammonium phosphate in 50% methanol was used as an intermediate wash step. The analytes were eluted from the plate with 1 mL 10% ammonium hydroxide in acetonitrile. The eluate was evaporated to dryness under nitrogen at 40 °C and reconstituted in 1 mL mobile phase.

The comparison of the L/L and SPE methods can be seen in Figure 6. Note that a significantly cleaner extract was achieved using the SPE method, which removed almost all of the early-eluting matrix interferences and enabled a higher analyte recovery.

Conclusion

Astec CHIROBIOTIC V2, with the vancomycin chiral selector, provided rapid, baseline resolution of the enantiomers of methamphetamine and amphetamine. Since the compounds are not isobaric, the same method could be applied to the LC/MS analysis of all four enantiomers, should that be desired. The choice of acetic acid and ammonium hydroxide as mobile phase additives to methanol:water



in the polar ionic mode gave the optimal LOQ and highest resolution for a sensitive, high throughput chiral screening of amphetamine and methamphetamine, either individually or simultaneously. Sample preparation using Supel-Select SCX SPE removed many of the interferences from the urine matrix. The sample prep and LC/MS techniques provide a robust and easily transferrable method.

Figure 6. LC/MS Analysis of (+/-)-Methamphetamine from Urine on Astec CHIROBIOTIC V2 after L/L Extraction or SPE using Supel-Select SCX

L/L Extraction Conditions:

125 μ L spiked urine spiked at 125 ng/mL each analyte, add 1 mL diethyl ether, vortex 30 min, centrifuge 10,000 rpm for 10 min, remove 500 μ L aliquot of organic supernatant, evaporate to dryness under nitrogen at 55 $^{\circ}$ C, reconstitute in 500 μ L mobile phase, filter through 0.22 μ m PVDF filter

SPE Extraction Conditions:

matrix: 1 mL of synthetic urine spiked with 25 ng/mL of each analyte, acidified to 3-4 pH with formic acid

SPE well plate: Supel-Select SCX SPE 96-well Plate, 30 mg/well (575664-U)

conditioning: 1 mL 1% formic acid in acetonitrile, then 1 mL water

sample addition: 1 mL spiked urine

washing: 2 mL water, then 1 mL 25% methanol (for amphetamine 1 mL of 5 mM dibasic ammonium phosphate in 50% methanol was used as an intermediate wash step)

elution: 1 mL 10% ammonium hydroxide in acetonitrile

eluate post-treatment: evaporate to dryness under nitrogen at 40 $^{\circ}$ C, reconstitute in 1 mL mobile phase

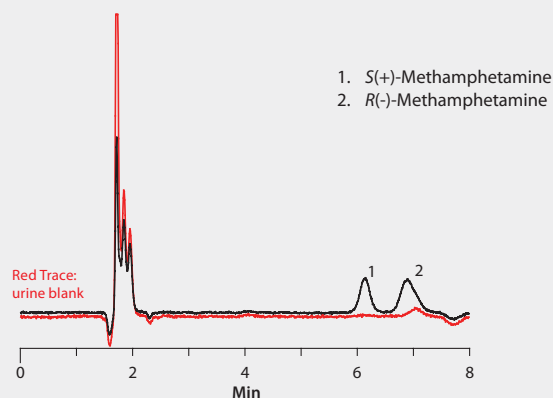
LC/MS Conditions Same as Figure 2 Except:

mobile phase: [A] methanol; [B] water; [C] acetic acid; [D] ammonium hydroxide; (95:5:0.1:0.02, A:B:C:D)

pressure: 1257 psi (87 bar)

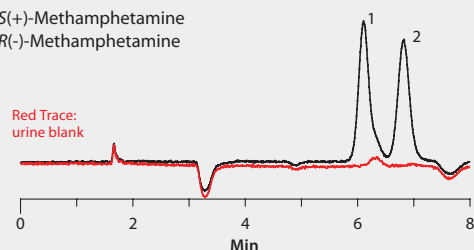
sample: extracted, spiked urine

L/L Extraction Method



Supel-Select SCX SPE Method

1. S(+)-Methamphetamine
2. R(-)-Methamphetamine



Featured Products

Description	Cat. No.
Sample Preparation	
Supel™-Select SCX SPE 96-well Plate, bed wt. 30 mg/well, pk of 1	575664-U
HPLC Columns	
Astec CHIROBIOTIC V2, 15 cm x 4.6 mm I.D., 5 μ m particles	15023AST
Astec CHIROBIOTIC V2, 25 cm x 4.6 mm I.D., 5 μ m particles	15024AST
Astec CHIROBIOTIC V2 Chiral HPLC Guard Column. 2 cm x 4.0 mm I.D., 5 μ m particles	15100AST
Supelguard™ Guard Cartridge Holder, stand-alone (swivel-type), for use with Supelguard cartridges (2 cm L x 2 to 4.6 mm I.D.)	21150AST
Mobile Phase Components	
Methanol, LC-MS Ultra CHROMASOLV®, tested for UHPLC-MS, 1 L, 2 L	14262
Water, LC-MS Ultra CHROMASOLV, tested for UHPLC-MS, 1 L, 2 L	14263
Acetic acid, eluent additive for LC-MS, 50 mL	49199
Ammonium hydroxide, $\geq$ 25% in water, eluent additive for LC-MS, 10 x 1 mL, or 100 mL	44273
Ammonium trifluoroacetate, for mass spectrometry, $\geq$ 99.0% (NT), 1 g	89895
Standards*	
Surine™ Negative Urine Control, 50 mL	S-020
($\pm$)-Amphetamine solution, 1.0 mg/mL in methanol, ampule of 1 mL, Cerilliant Certified Reference Material	A-007
S(+)-Amphetamine (dextro-Amphetamine) solution, 1.0 mg/mL in methanol, ampule of 1 mL, Cerilliant Certified Reference Material	A-008
R(-)-Amphetamine (levo-Amphetamine) solution, 1.0 mg/mL in methanol, ampule of 1 mL, Cerilliant Certified Reference Material	A-049
($\pm$)-Methamphetamine solution, 1.0 mg/mL in methanol, ampule of 1 mL, Cerilliant Certified Reference Material	M-009
S(+)-Methamphetamine solution, 1.0 mg/mL in methanol, ampule of 1 mL, Cerilliant Certified Reference Material	M-020
R(-)-Methamphetamine solution, 1.0 mg/mL in methanol, ampule of 1 mL, Cerilliant Certified Reference Material	M-024

* Deuterated CRMs of methamphetamine and amphetamine are also available.

For additional information, visit

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