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Review

Determination of methamphetamine enantiomer ratios in urine by gas chromatography–mass spectrometry☆

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ABSTRACT

Analysis of the enantiomers of methamphetamine and its metabolite amphetamine is an extremely important process for a number of scientific disciplines. From studies of biological activity and mechanisms through determination of precursor molecules in a criminal investigation are all served by this analytical procedure. Utilization of gas chromatography–mass spectrometry with chiral derivatizing reagents is the most common chiral procedure and produces excellent results. Of the chiral derivatizing reagents available, the most widely used is trifluoroacetyl-*l*-prolyl chloride (TPC). Utilization of other derivatives require either synthesis by the analyst or have not shown themselves to provide as good a separation as did the TPC reagent.

Use of chiral stationary phases yield good results but the disadvantages of temperature limits of these columns and the narrow use to which the columns can be put has limited their utilization. A significant utility of the chiral stationary phase is the ability to determine the purity of a chiral derivatizing reagent. Even if not used for routine analysis of enantiomers, utilization of this procedure can determine the purity of a reagent such as TPC and allow for accurate calculation of actual amounts of each enantiomer. This can be estimated using chiral derivatives on an achiral column, but it is limited to the extent that it is not able to differentiate enantiomeric impurity in the reagent *versus* the drug itself. Description of chromatographic procedures primarily focusing on gas chromatographic–mass spectrometric techniques but also including liquid chromatographic techniques along with examples of extraction and derivatization procedures is the focus of this review.

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LIST OF ABBREVIATIONS

CAS No.	Chemical Abstracts Service Number
DB-1	Cross-linked methylsilicone
DB-5	5% Phenyl methylsilicone
DB-17	50% Phenyl methylsilicone
DNBPG	(*R*)-N-(3,5-Dinitrobenzoyl)phenylglycine
ED	Electrochemical detector
FID	Flame ionization detector
FMOC	Fluorenylmethyl chloroformate
GC	Gas chromatography
GC–MS	Gas chromatography–mass spectrometry
GITC	2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate
HFBA	Heptafluorobutyric anhydride
HPC	Heptafluorobutyryl-*l*-prolyl chloride
HPLC	High-performance liquid chromatography
IR	Infrared
LC	Liquid chromatography
LC–MS	Liquid chromatography–mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MTPA	α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride
NMR	Nuclear magnetic resonance
NPD	Nitrogen–phosphorus detector
NPSP	4-Nitrophenylsulfonyl-*l*-prolyl chloride
MS	Mass spectrometry
PFPA	Pentafluoropropionic anhydride
PPC	Pentafluoropropanyl-*l*-prolyl chloride
SIM	Selected ion monitoring
S/N	Signal-to-noise ratio
TFA	Trifluoroacetic anhydride
TLC	Thin-layer chromatography
TPC	Trifluoroacetyl-*l*-prolyl chloride
ZPC	N-Benzyloxycarbonyl-*l*-prolyl chloride

Note regarding nomenclature of enantiomers

In the literature, various terms are used to describe the same chemical structure. Below is a list of terms which are used in the references cited in this review grouped by their common definition. Structures of methamphetamine enantiomers as described below are shown in Fig. 1.

d *d*-Stereoisomer of a compound;
D D-Stereoisomer of a compound;
S *S*-Stereoisomer of a compound;
(+) (+)-Stereoisomer of a compound; represents the chemical structure which rotates plane-polarized light to the right.

d-, D-, (*S*)-, (+)- and dextro(meth)amphetamine refer to the same isomer.

l *l*-Stereoisomer of a compound;
L L-Stereoisomer of a compound;
R *R*-Stereoisomer of a compound;
(−) (−)-Stereoisomer of a compound; represents the chemical structure which rotates plane-polarized light to the left.

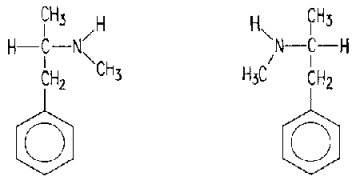
l-, L-, (*R*)-, (−)- and levo(meth)amphetamine refer to the same isomer.

1. INTRODUCTION

The analysis of amphetamines as a drug class has been a major part of drug and toxicological analysis for many years. This drug class encompasses a number of different compounds, but the most widely abused are amphetamine and methamphetamine. These drugs have been widely available for many years and their abuse has a history essentially as long as the drugs themselves. Because of their long history of use, a significant amount of the investigative work with amphetamines was completed many years ago and there have been several extensive reviews and texts written on their analysis and metabolism [1–3].

Optical isomers, as the term implies, are isomers that give rise to different optical activity which is classically measured using some optical technique. The common designation assigned to a compound for this unique form of isomerism also derives from optical measurements relating to the rotation of plane-polarized light either to the right (*d*, +) or to the left (*l*, −) when it passes through a solution containing the isomer. The structural orientation of the asymmetric group is also often designated using the letters D and L or *R* and *S*. Structures of methamphetamine isomers are shown in Fig. 1. Light-measurement techniques are not commonly employed in the routine analysis of amphetamines for a number of reasons particularly related to sensitivity. The ability to measure these optical isomers is an important function, however, and is one that has largely been taken over by chromatographic techniques when dealing with samples of biological origin.

Analysis of optical isomers of the amphetamines, or more precisely in this instance enantiomers, has a history going back to the 1960s and draws heavily on work initially developed for the analysis of amino acids [4]. From this work came the ability to separate compounds whose only difference was the orientation of atoms around a central carbon. The utility of this technique has reached into many areas of



D - Methamphetamine L - Methamphetamine

Fig. 1. Structures of methamphetamine showing enantiomeric orientation of the molecules.

chemistry, biochemistry and pharmacology and has led to substantial understanding of the workings of the human body.

The differences in biological activity of the enantiomers of a drug molecule are widely used by the pharmaceutical industry in the development of drugs that have very specific function and minimize undesirable side-effects. In the forensic science community, the ability to differentiate enantiomers of drugs, and methamphetamine in particular, is of significance. Both amphetamine and methamphetamine have legitimate medical uses, but their abuse potential is very high. The Vicks inhaler, an over-the-counter medication in the USA, is an "exempted product". This means that although the product contains 50 mg of L-methamphetamine (labeled under the pseudonym desoxyephedrine), it is exempted from control and can therefore be freely purchased without prescription. As it does in fact contain methamphetamine, there are several forensic toxicological situations where knowing the enantiomer present in a biological sample is critically important in order to interpret results properly. One such situation is in the area of urine drug testing which has gained wide use in many sectors, both public and private, and its use continues to expand. If a sample is analyzed and found to contain methamphetamine, it is important to determine whether or not the drug came from a controlled or over-the-counter source. This can also be an important question in *post mortem* analysis of body fluids and tissues to help determine whether or not the deceased was influenced in any way by drugs or if the drug played any role in the cause of death. This information is important owing to the significant differences in pharmacological activity and effect of the different enantiomers.

From a biochemical standpoint, investigation of the metabolism of drug enantiomers is significant in understanding areas ranging from membrane receptors to active sites on metabolic enzymes and from absorption to excretion. Use of enantiomers of amphetamine, methamphetamine and related N-substituted alkylamines has given great insight into the workings of the human body, particularly in the nervous system.

Separation of enantiomers can be accomplished by either of two fundamental processes. These are sometimes referred to as direct and indirect separation, both of which have their advantages and disadvantages. The direct method involves analysis of the enantiomers in the chromatographic system using a chiral stationary phase. In this instance the enantiomers form transient diastereomers with the stationary phase and their retention in the column is dependent on the strength of that interaction. The indirect method involves reaction of the enantiomers with a chiral derivatizing reagent to form diastereomers prior to their application to the chromatographic system. After conversion to the diastereomer, the separation can be accomplished using standard achiral stationary phases. These methods have been successfully applied to both gas (GC) and liquid chromatography (LC).

2. SAMPLE PREPARATION

Preparation of a sample to determine the presence of amphetamines can be a relatively simple process. The amine group has a relatively high pK value and simply making the solution alkaline readily converts the amine group from the very polar $-NH_3^+$ to the far less polar $-NH_2$ form. This loss of hydrophilic character coupled with the already hydrophobic nature of the remainder of the molecule makes it easily extracted into a non-polar organic solvent. This process can be accomplished in a few minutes and the sample can be ready for analysis by a variety of analytical procedures, including sophisticated techniques such as gas chromatography–mass spectrometry (GC MS). Unfortunately, there are a number of other biological molecules that are chemically related to the amphetamines which are also extracted during a process as simple as that just described. This is true of all biological tissues and fluids, including the aqueous environment of a urine sample.

A wide variety of extraction procedures are available for the identification and determination of amphetamines and virtually all that produce good results for those purposes are also accept-

able for the analysis of enantiomers. The only additional consideration when analyzing enantiomers is the fact that the chromatograms become more complex when chiral separation is involved. Each compound present will give a separate peak for each enantiomer, thereby increasing (potentially doubling) the complexity of the chromatogram. As a result, the possibility of different compounds co-eluting is far greater than with standard analysis where enantiomeric pairs elute as a single peak.

In order to overcome some of the problems of the analysis of complex mixtures within a sample, extraction schemes have been developed using liquid–liquid and solid-phase extraction techniques. The description of several of these techniques is the topic of the next two subsections and serves as a prelude to the actual analysis of the samples. Because extraction for enantiomeric analysis is essentially the same as that for standard analysis, only a selected few of the many different approaches are discussed.

2.1. Liquid–liquid extraction

The simplest extraction reported for the amphetamines is also one of the earliest and has been applied in many different laboratories. It involves adjustment of the pH to greater than the pK of the amine group, which makes the molecule far less water-soluble. This is accomplished by addition of base to raise the pH to > 10. Chloroform is then added, the sample is vortex-mixed for a short time and a small amount of the chloroform is injected into the gas chromatograph. Although some minor variations exist, a good example of this procedure was described by Fitzgerald *et al.* [5]. Their extraction started with addition of 0.1 ml of 12 M NaOH to a 2-ml urine sample followed by 0.1 ml of chloroform. Following centrifugation to separate the phases completely, 3 μl of the chloroform were injected into the GC–MS instrument. This method is similar to those described by other investigators [6]. Significant amounts of other compounds are also extracted in this manner and in an attempt to help isolate the amphetamines, additional extraction steps are incorporated to further separate the amphetamine and methamphetamine from related compounds. With the addition of additional extraction steps, the yield, or extraction efficiency, is typically decreased but the elimination of related, and potentially interfering, compounds often outweighs the loss of recovered drug.

An example of a process that involves extraction of the drug from a basic solution followed by another extraction was described by Czarny and Hornbeck [7]. In their procedure, the urine was made basic by the addition of NaOH and the drug was extracted into dichloromethane by shaking for 30 min and then centrifuging to separate the layers. The organic phase was made acidic by the addition of 2 ml of 0.15 M H_2SO_4, shaken for 15 min then centrifuged. The drug, now in the aqueous layer, was transferred into another tube where the solution was made basic again by addition of NaOH (1 ml of 1.0 M solution) followed by addition of 1-chlorobutane. The tubes were vortex-mixed and the phases separated by freezing the aqueous layer in an dry-ice–2-propanol bath and pouring off the organic layer. This method is more complex than the first one described and illustrates a common method to clean up the original extract by back-extracting to separate the drugs of interest from other compounds. This is accomplished by changing the pH so the amine group is again charged and extracted from the organic phase into the acidic solution. This leaves behind any of the organic compounds originally extracted which are not ionized in the process of adding the acid and therefore remain in the organic solvent. The process is continued by making the solution basic with the addition of NaOH and extracting the amphetamines into 1-chlorobutane, followed by derivatization and evaporation. Many liquid extraction procedures are based on this principle but vary in the organic solvent, incubation times, etc.

Another technique used to minimize evaporative losses is to add HCl in the process of acidification, thus forming the hydrochloride salt, which has advantage of not being lost as readily

during the process of solvent evaporation, a process where substantial amounts of the amphetamines can be lost. An alternative is to use a "keeper" solvent that has a boiling point above the temperature used in the evaporation, thereby diminishing the evaporative loss of the amphetamines. These considerations are important for the analysis of amphetamines in general, but because when isolating the enantiomers the amount of each individual enantiomer is obviously lower, minimizing losses during extraction and derivatization is important for accurate measurements. This is of particular concern when the proportion of one enantiomer is only a small percentage of the total.

2.2. Solid-phase extraction

Solid-phase extractions have become popular owing to the relative simplicity of the procedure. Another reason is that with multiple-step extraction procedures, the time and solvent used are often less than for comparable liquid–liquid extraction methods. Manual manipulation of the sample is also generally less with solid-phase extractions and it is therefore easier to develop and conduct the extraction in a consistent manner.

An extraction procedure utilizing a solid-phase technique involved the Extrelut 20 system (Merck, Rahway, NJ, USA). Although the overall results from this procedure were acceptable, the entire process took far too long to be of practical use. It involved derivatization which took overnight to complete (a consequence of the reactivity of the derivatization reagent) and large solvent volumes (40 ml of diethyl ether) to extract the drug from the column [8].

Taylor *et al.* [9] used Detectabuse extraction columns (Biochemical Diagnostics, Edgewood, NY, USA) to extract amphetamine and methamphetamine from urine. After preparation of the column using methanol followed by 1 *M* phosphate buffer (pH 6.0), a 5-ml urine sample to which 2 ml of the phosphate buffer had been added was poured on to the columns and drawn through with a slight vacuum. The top cotton plugs, used to prevent particulate matter from plugging the column, were removed and the column was washed with distilled water followed by 2-propanol–water (25:75) and finally hexane. The drugs were then eluted by addition of 1% HCl in methanol. Recoveries of amphetamine and methamphetamine by this method were 78 and 87%, respectively. This extraction not only proved to be rapid, but also showed no interference from related compounds such as phenylpropanolamine, ephedrine and phentermine.

The method described above, with minor modifications, is available for various extraction cartridges. Virtually all manufacturers have made available extraction procedures for amphetamines to be used with their products. All of these procedures can give acceptable results for extraction of amphetamines from urine.

3. DERIVATIZATION AND GAS CHROMATOGRAPHY

Derivatization and GC are two separate and distinct processes, but the impact of one on the other makes separation of the discussion almost impossible. For this reason, reviews of both processes are combined in this section.

Once extracted from the biological matrix, the amphetamines are often analyzed without further processing. In the analysis of the D- and L-enantiomers, derivatization is commonly used to impart chromatographically unique characteristics to allow for their separation. This is accomplished by using an enantiomerically pure derivatizing reagent which itself has an asymmetric center. In this instance, the combination of derivative and drug forms a diastereomer. The conformation of the diastereomers formed in this way is sufficiently different that they can be chromatographically separated using standard archiral stationary phases.

An alternative to derivatization with a chiral derivatizing reagent is the use of a chiral stationary phase in the chromatographic system, which can also serve to separate the enantiomers. Although derivatization is not required for separation if a chiral stationary phase is used, it can be used to improve the GC behavior of the enantiomers.

3.1. Gas chromatography of chiral derivatives

A number of chiral derivatives have been successfully applied to the analysis of amphetamine and methamphetamine enantiomers. The most commonly used derivatizing reagent is trifluoroacetyl-*l*-prolyl chloride (TPC). The probable reason for the popularity of this reagent is its commercial availability. Procedures for the synthesis of this and other chiral derivatizing reagents are available [10] and can be accomplished in any analytical laboratory. Although not technically difficult for a competent chemist, the synthesis requires reasonable care. For example, during synthesis temperature control is critical and even mild elevation can yield a mixture of enantiomers of the derivative [10,11], which makes the accurate determination of enantiomeric composition of the target analyte difficult or impossible.

TPC has been used in a number of studies to separate the enantiomers of various amphetamines. The early work with the assay provided much valuable information but the GC suffered primarily from the lack of resolving power and sensitivity of packed columns. Studies in which capillary columns were used clearly demonstrated better separations than did those in which packed columns were used. In addition, the enantiomeric purity of the reagents in some of the early studies was not high. The evolution of the use of TPC in the resolution of enantiomers is long and varied, but in the separation of amphetamines begins with some of the initial work of Gordis [12] and Gunne [13], who used the reagent with considerable success and laid the foundations for subsequent work. The procedure was further improved through the work of Wells [10], who made significant strides in improving the purity of the reagent. His refinement of the preparation of the reagent made a significant difference, leading to preparations that showed as high as 99% enantiomeric purity.

On-column derivatization has been used in the qualitative and quantitative analysis of amphetamines by a number of investigators [5,6,14]. Extending that principle, Fitzgerald *et al.* [5] used TPC to derivatize amphetamine and methamphetamine on-column in order to separate the enantiomers. A 3-μl volume of urine extract in chloroform was drawn into a 10-μl syringe, followed by 3 μl of the TPC reagent. The extract and derivatizing reagent were then injected into the GC–MS instrument. The injection port temperature was set at 250°C with a splitting ratio of 10:1. The oven temperature was set initially at 190°C for 4 min, then increased at 20°C/min to a final temperature of 250°C. This led to the separation of methamphetamine enantiomers in less than 6 min on a 12-m DB-1 equivalent column (Fig. 3). This method of derivatization offers several advantages, including speed of analysis, as derivatization is typically accomplished prior to injection which takes, depending on the method, more than 1 h when considering the derivatization followed by elimination of the excess of reagent and evaporation. It also allows the same extract to be used for determination of the drug either underivatized or by on-column derivatization using an achiral derivative followed by separation of the enantiomers using a chiral derivative rather than preparation of separate extracts and derivatives for each analysis. The process also has disadvantages, which include the possibility of incomplete derivatization or variable derivatization depending on the amount of material in the injection port at the time. Theoretically, incomplete derivatization could be a problem when the derivatization takes place in a reaction tube, but in typical procedures the reaction times are sufficient to ensure that this is not a problem. Although different degrees of derivatization can be critical for quantitative analysis, use of a deuterated internal standard minimizes this concern. Differing rates for the derivatization of the enantiomers is also a potential problem, but with methamphetamine it has been shown that the enantiomers do not react at rates different enough to be of concern [5]. Although the enantiomers were essentially baseline resolved, the authors commented that when one of the enantiomers was present in small amounts, it would appear as a shoulder on the main peak which sometimes did not integrate separately. Further separation of the peaks would overcome

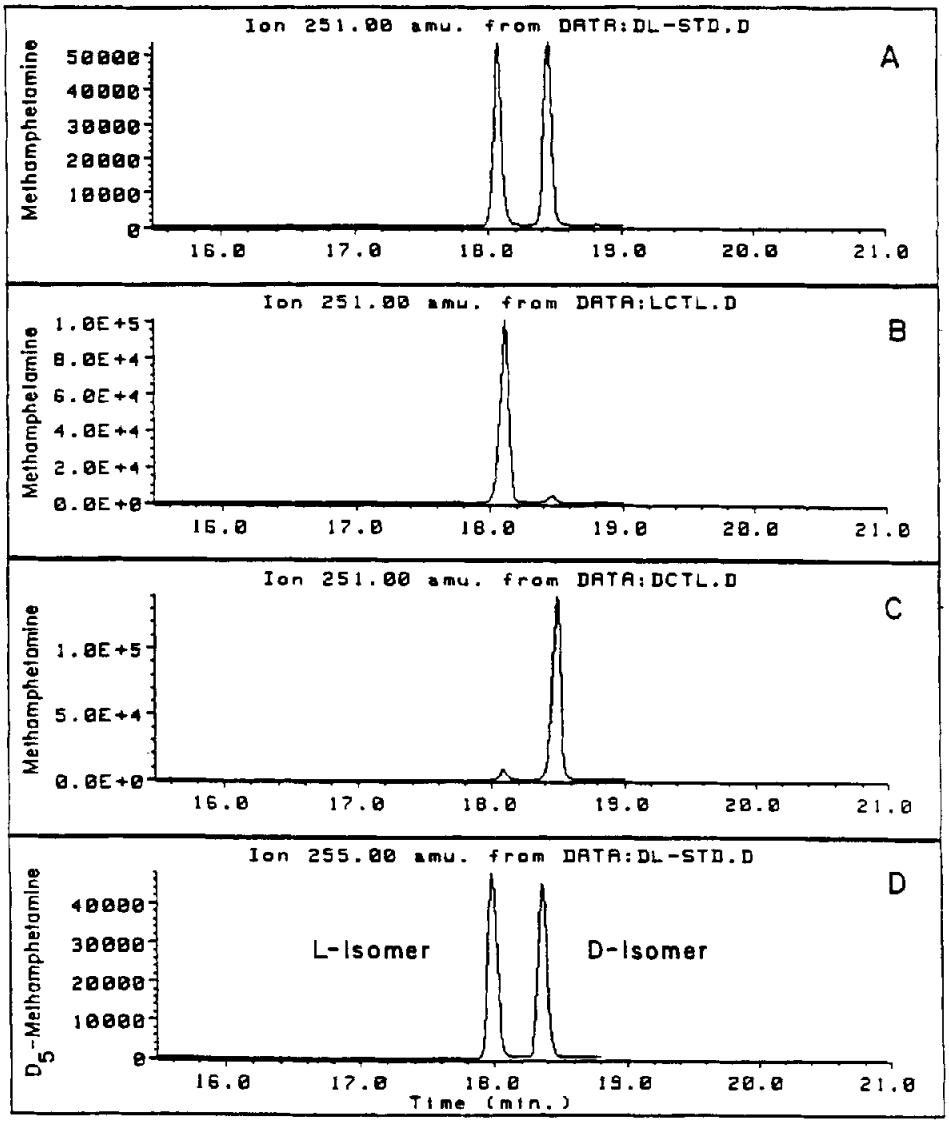


Fig. 2. Base peak ion chromatograms for methamphetamine isomer analysis. All compounds 500 ng/ml. (A) *d,l*-Methamphetamine; (B) *l*-methamphetamine; (C) *d*-methamphetamine; (D) *d,l*-d_5-methamphetamine (internal standard). GC conditions: 120°C for 2 min; 4°C/min to 190°C.

this problem but would necessitate increasing the retention time (see Fig. 2).

Although the use of the trifluoroacetyl group in combination with *l*-proline is the most common, other fluorinated groups have been used to create a chiral derivative. In a fairly comprehensive survey of amino acids and perfluorinated acyl groups, Souter [15] evaluated the utility of a variety of different amines including *l*-proline, *l*-leucine, *l*-valine and *l*-alanine coupled with trifluoroacetyl, pentafluoropropionyl and heptafluorobutyryl groups. One of the clear results from his evaluation was the fact that the best separation depended on a number of factors and the best choice of derivatizing reagent depended to a large extent on the particular amine under investigation.

Reaction of heptafluorobutyric anhydride with

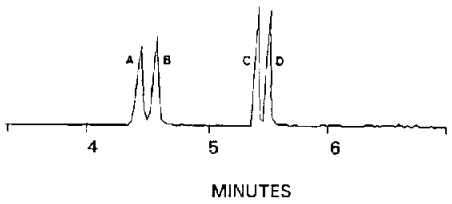


Fig. 3. Chromatogram of trifluoroacetyl-*l*-prolyl chloride-derivatized (A) (−)-methamphetamine and (B) (+)-methamphetamine separated on a 12-m DB-1 equivalent column. Other peaks are methoxyphentamine (internal standard). GC conditions as described in the text. (From ref. 5, with permission of Preston Publications, a division of Preston Industries, Inc.)

l-proline to form heptafluorobutyryl-*l*-prolyl chloride (HPC) has been used for the separation of a number of amphetamine-related compounds [16–18]. Srinivas *et al.* [19] used HPC to derivatize twelve amphetamines on an OV-225 capillary column with temperatures ranging from 190 to 250°C using electrochemical detection (ED). All of the target amines were well resolved with run times of less than 14 min.

(−)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA) has been used to separate amphetamine and methamphetamine enantiomers. Initial development of this reagent was due to Dale *et al.* [20] and it was evaluated in a comparative study by Gal [21]. In the latter study, the utility of MTPA in the enantiomeric separation of a number of different amines, including amphetamine and methamphetamine, was evaluated. Derivatization was carried out by mixing the amine with 1,2-dichloromethane, MTPA and pyridine and heating at 70°C followed by immersion into an ice-bath. Under these conditions, amphetamine derivatives were well separated on 3% phenylmethylsilicone on a dimethylchlorosilane-treated diatomite support. Methamphetamine was essentially not derivatized, however. Mori *et al.* [8] used the same derivative and were successful in the derivatization of methamphetamine and amphetamine and both were well separated by GC. The conditions used to derivatize methamphetamine were extensive and involved addition of the derivatizing reagent and pyridine to the extracted amine followed by overnight incubation. The derivatives in this instance are also useful for analysis by nuclear magnetic resonance (NMR), which was one advantage of using this process. Under ordinary circumstances, however, the difficulty in derivatization of methamphetamine with this compound makes it an unlikely choice.

A number of other derivatizing reagents have been used, including N-(*R*)-α-phenylbutyramides, N-(*S*)-α-phenylpropionamides and N-(*R*)-α-chlorophenylacetamides [22]. This study showed that all of the derivatizing reagents gave acceptable results, but the N-(*R*)-α-chlorophenylacetamides were the least effective with no separation of methamphetamine and only a poor separation of amphetamine. The most effective results for methamphetamine were seen with the N-(*R*)-α-phenylbutyramides, which gave excellent separation on both SE-30 and OV-17 columns. N-(*S*)-α-phenylpropionamides were slightly less effective with methamphetamine and did not separate amphetamine on the SE-30 column and was only slightly better on OV-17.

Hughes *et al.* [23] recently used (−)-menthyl chloroformate as the derivatizing reagent. This method showed excellent quantitative linearity over the range of 50–6000 ng/ml. The separation and identification of methamphetamine isomers were not effected by other commonly encountered compounds such as ephedrine, pseudoephedrine, phentermine, phenylpropanolamine or 2-phenylethylamine. Separation on two different stationary phases was described. In the one instance, a DB-5 column was used at 235°C (isothermal) and showed no interference from related compounds, but the methamphetamine enantiomers, although separated, were not baseline resolved. In addition, the retention time was *ca.* 31 min. Changing the polarity of the stationary phase by using a DB-17 column and using temperature programming more than halved the retention time and improved resolution (Figs. 4 and 5). Unfortunately, neither of these procedures was able to separate the enantiomers of amphetamine.

The major interest in the enantiomeric forms of methamphetamine in a forensic context is associated with the potential of the L enantiomer to

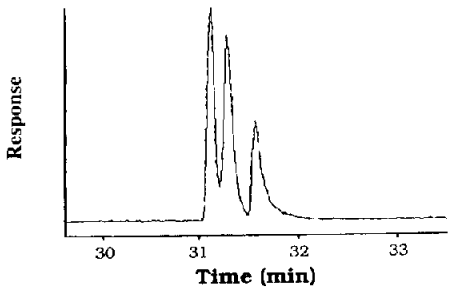


Fig. 4. Total ion chromatogram of (+)-menthyl chloroformate derivatives of amphetamine, (+)-methamphetamine and (−)-methamphetamine separated on a 15-m DB-5 column. Elution order: (+)-methamphetamine, (−)-methamphetamine, amphetamine. GC conditions as described in the text. (From ref. 23, with permission of Preston Publications, a division of Preston Industries, Inc.)

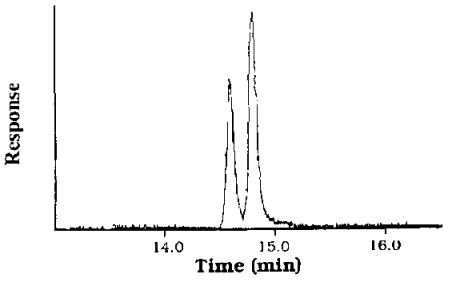


Fig. 5. Total ion chromatogram of (−)-menthyl chloroformate derivatives of (+)-methamphetamine and (−)-methamphetamine separated on a 15-m DB-17 column. Elution order: (+)-methamphetamine, (−)-methamphetamine. GC conditions as described in the text. (From ref. 23, with permission of Preston Publications, a division of Preston Industries, Inc.)

be present in the over-the-counter Vicks inhaler. Owing to the way in which methamphetamine enantiomers are metabolized and excreted, evaluation of the enantiomer composition of amphetamine can be critical in the evaluation of methamphetamine data, particularly toward the end of the excretion profile. For this reason, the inability of this procedure to separate amphetamine enantiomers is a significant limitation if a mixture of methamphetamine enantiomers was ingested.

In an interesting variation, Singh *et al.* [24] used enantiomerically pure amphetamine to derivatize a number of non-steroidal anti-inflammatory arylalkanoic acids. In this instance it was the amphetamine that was used to derivatize the compounds of interest and effect their separation on an achiral GC. Use of either D- or L-amphetamine worked well for the separation of these compounds and offered a resolution similar to those of previously reported methods while offering some advantages of stability.

3.2. Direct gas chromatography of enantiomers

A number of achiral stationary phases have been used in combination with a chiral derivatizing reagent to separate enantiomers. In these instances, the column is less significant than the derivatizing reagent as the separation is based on the diastereomer formed from the combination of the drug and the derivative. The nature of the column in these instances is concerned primarily with adequate separation of the derivatized enantiomers and separation of the drug of interest from other potentially interfering compounds.

Chiral stationary phases take a slightly different and more direct approach to the separation of enantiomers. Rather than creating a diastereomer from a chiral derivatizing reagent and the enantiomer, the compound is passed through a column containing a chiral stationary phase with which the drug interacts. This interaction is transient and therefore each enantiomer will interact differently with the stationary phase. The better the fit between the stationary phase and the enantiomer, the longer the compound is retained in the column. The difference in interaction between each of the enantiomers and the stationary phase accounts for the separation of the enantiomers from each other. This approach does not require derivatization of the drugs, which can make the preparation and analysis time substantially shorter than taking each extract through a derivatization, evaporation and reconstitution sequence. In some instances, however, the drug is derivatized to improve the chromatographic behavior.

Several significant studies using a chiral stationary phase coupled with a chiral derivatizing

reagent showed the significance of both methods [25,26]. A clear demonstration of the impurity of the derivatizing reagent was shown and the impact of that impurity was discussed. In most instances, the amount of the impurity was small enough that it could be easily accounted for in normal analysis once the purity of the reagent had been initially demonstrated. As one would expect, the combination of a chiral derivatizing reagent (TPC) with a chiral stationary phase (Chirasil-Val) gave rise to the possibility of four peaks, representing: the *d*-isomer of the drug and the *l*-isomer of the derivatizing reagent; the *d*-isomer of the drug and the *d*-isomer of the derivatizing reagent; the *l*-isomer of the drug and the *l*-isomer of the derivatizing reagent; and the *l*-isomer of the drug and the *d*-isomer of the derivatizing reagent. These peaks were well separated for the enantiomers of amphetamine but not for methamphetamine. Under the conditions described, methamphetamine gave rise to only three separate peaks. The inability of the system to separate the mixture of TPC and methamphetamine into four separate peaks was attributed to the substitution of the active hydrogen on the amine nitrogen of amphetamine with a methyl group in methamphetamine. Once the enantiomeric purity of the derivatizing reagent has been determined, mathematical calculations can be made on the raw data to account for the impurity. Although this will give an acceptable result, the possibility that the derivatizing reagent might change (racemize) makes it important to analyze a control sample to determine if the calculations are still valid.

4. MASS SPECTROMETRY

Mass spectrometry (MS) is virtually universally recognized as the most specific and sensitive detection method for a chromatographic system [27]. The ability to examine not only the retention time of a compound through the chromatographic system but also the characteristic ion fragmentation pattern by MS makes the latter a powerful analytical tool. Unequivocal identification of isomeric compounds is not always easily accomplished with the mass spectrometer, however. For example, for the structural isomers methamphetamine and phentermine, the electron ionization mass spectra of the two compounds are essentially identical. For those two isomers, the differentiation comes from the chromatographic retention time differences rather than mass spectral differences. With enantiomers, however, separation is not accomplished using most chromatographic columns and conditions. The mass spectra of enantiomers are also indistinguishable from each other, leading to the necessity of either changing to another analytical method or separating the enantiomers chromatographically to ensure proper identification. This can be accomplished by use of a chiral derivatizing reagent or by use of a chiral stationary phase in the chromatographic system as described previously.

One of the most common derivatizing reagents used for the separation of amphetamine and methamphetamine isomers is TPC. Unfortunately, this reagent yields mass spectra which are not ideal (Fig. 6). The base peak of the spectra (*m/z* 166) arises from the derivatizing reagent itself and is therefore not characteristic of the compound being analyzed (see Fig. 6). It also does not yield other masses of high abundance which would allow for consistent ion ratio calculations across a wide concentration range except for low *m/z* values common to many different compounds. For this reason, many laboratories identify amphetamine and methamphetamine using an achiral derivative which gives more characteristic spectra for the qualitative and quantitative identification of the drugs and use a second derivative (*e.g.*, TPC) for determination of the enantiomeric composition. In these instances, the identification of the enantiomeric forms is accomplished using perhaps one or two ions rather than the three which are generally required for positive identification of a compound. Given that the compound has independently been identified by a GC–MS method, using only one or two ions to demonstrate the enantiomeric composition of methamphetamine in a sample is well accepted even in forensic work.

Analysis of the enantiomers using chiral sta-

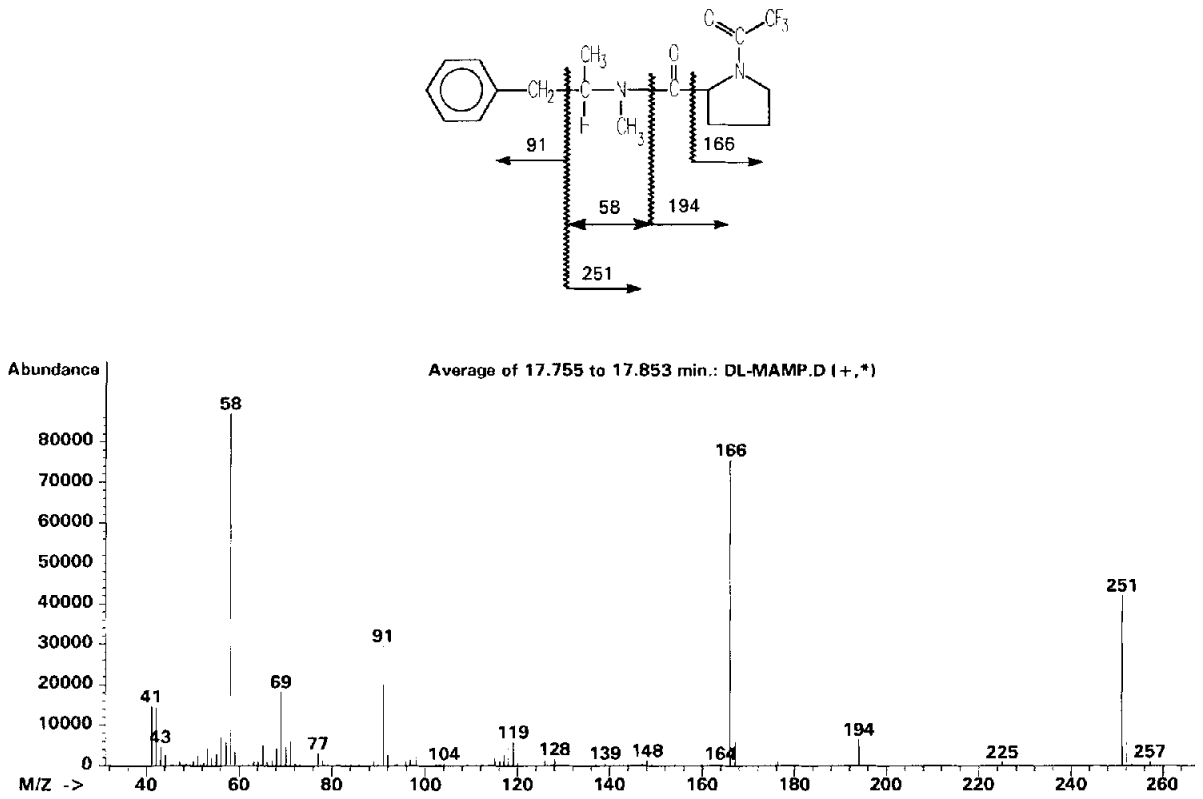


Fig. 6. Electron ionization mass spectra for the trifluoroacetyl-*l*-prolyl derivative of methamphetamine showing the structure of derivatized molecule and major ion fragments.

tionary phases can have a substantial effect on the mass spectra from methamphetamine. If the compound is not derivatized, the spectrum is very simple and has only one characteristic but low-mass ion (m/z 58). The derivatization of methamphetamine, however, not only improves the chromatographic peak shape but also leads to a more complex and characteristic spectrum.

Using LC–MS in the filament-on mode, methamphetamine enantiomers were identified with high-mass ions of the 2-naphthyl chloroformate derivative [28]. This method, described later in this review, produced a number of high-mass ion fragments which are useful in the monitoring and identification of the compounds.

5. ALTERNATIVE TECHNIQUES

GC and GC–MS are the most widely used techniques for the analysis of amphetamine and methamphetamine enantiomers. Other techniques have also been applied successfully to the same analysis. The most popular of these alternative procedures is high-performance liquid chromatography (HPLC). Another less frequently encountered alternative is thin-layer chromatography (TLC), which has also been used successfully to separate drug enantiomers.

5.1. High-performance liquid chromatography

A number of different stationary phases and detectors have been used in the analysis of drug enantiomers. As with GC, analysis of enantiomers of the amphetamines by HPLC includes both chiral derivatization and use of chiral stationary phases. Detectors used include the UV spectrophotometer, which is the most common,

and fluorescence and mass spectrometers. Amphetamines themselves absorb in the UV range but must be derivatized to be detected by fluorescence. Use of ED is also possible for the detection of amphetamines.

5.1.1. Chiral derivatives

HPLC of amphetamines and related compounds using various derivatives includes the work by Noggle and Clark [29], who described the separation of amphetamine, methamphetamine, ephedrine and pseudoephedrine enantiomers using 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) as the derivatizing reagent. Tetrahydrofuran–water (3:7) was used as the mobile phase on a C_{18} column at a flow-rate of 1.5 ml/min. This resulted in an excellent separation of methamphetamine, ephedrine and pseudoephedrine enantiomers. A different mobile phase of tetrahydrofuran–water–acetic acid (35:70:1) was used with the same column and yielded good resolution of methamphetamine, ephedrine and pseudoephedrine enantiomers in about 30 min, although the resolution of the methamphetamine enantiomers was not to the baseline [30]. Amphetamine, however, proved to be more difficult to separate and eluted as a single peak under those conditions. This difficulty of separating amphetamine enantiomers was also seen with another chiral derivatizing reagent, 4-nitrophenylsulfonyl-*l*-prolyl chloride (NPSP) [31]. In that study, the mobile phase was changed from tetrahydrofuran–water to methanol–water (1:1), which then gave a separation, although not to the baseline, of the amphetamine enantiomers.

The elution order of the enantiomers under these conditions was D followed by L. With ephedrine and pseudoephedrine, the L-enantiomer eluted before the D-enantiomer. Although this separation of methamphetamine was effective, the retention time was *ca.* 40 min. With methanol–water, the retention time of amphetamine enantiomers was slightly over 20 min. Although the racemic mixture was easily interpreted, it appeared that if the percentage of either of the enantiomers was low, it would be difficult to determine whether or not it was indeed present.

Without question, the simplest method of sample analysis is to take the sample itself and analyze it directly. Using a polymer of fluorenylmethylchloroformate-*l*-proline (FMOC), Gao and Krull [32] analyzed enantiomers of amphetamine and several related compounds. Commercially available FMOC was converted into a polymeric form and placed in a stainless-steel reaction column [33]. A 10-μl urine sample was injected, after a simple filtration step, into the reaction column with a retention time of 5 min at 60°C, then into a C_{18} reversed-phase separation column using 40–48% acetonitrile–water as the mobile phase. The enantiomers of amphetamine were separated in *ca.* 30 min and detected with both UV and fluorescence detectors. Excellent quantitative results were obtained with this procedure over several orders of magnitude of concentration. Detection of as little as 1.1% of an enantiomer was demonstrated using this method.

5.1.2. Chiral stationary phases

Acetate derivatives of methamphetamine enantiomers were separated on OB and OJ Chiral-Cel (Daicel Ind. Co., Japan) stereoisomer analytical columns connected in series using *n*-hexane–2-propanol (9:1) at a flow-rate of 1 ml/min as the mobile phase [34]. The origin of the sample tested using this procedure was the hair of suspected methamphetamine abusers rather than a biological fluid, but the chromatographic technique is independent of the origin of the sample. This study is of note because the separation was significantly improved when the temperature was elevated from room temperature to 50°C. This method was also reported to give excellent quantitative results.

Hayes *et al.* [35] used both Regis Pirkle covalent phenylglycine and Regis Pirkle ionic phenylglycine columns in addition to a chiral derivatizing reagent. In this instance, TPC was employed as the derivatizing reagent. This combination gave excellent separation of the enantiomers with both stationary phases, although a slightly better separation was achieved with the ionic column.

5.2. Liquid chromatography–mass spectrometry

Virtually any of the techniques developed for HPLC can be used with liquid chromatography–mass spectrometry (LC–MS) provided that the solvent can be separated from the target analyte with currently available interfaces. The most common detector for the analysis of the amphetamines in HPLC is the UV spectrophotometer. Although UV detection is a common technique and has many positive aspects, it does not share the same capability as MS to positivily identify a compound. This limitation also extends to other HPLC detectors such as fluorescence and electrochemical detectors.

Several examples of LC–MS of methamphetamine enantiomers include the work of Lee *et al.* [28], who used the 2-naphthyl chloroformate derivative on a DNBPG [(*R*)-N-(3,5-dinitrobenzoyl)phenylglycine] column and a mass spectrometer in the filament-on mode with a thermospray interface. This method allowed the identification of a low percentage of the minor enantiomer peak (about 1%), which is an improvement over many of other LC techniques. Both the Regis Pirkle covalent and ionic phenylglycine columns were used to separate TPC-derivatized methamphetamine as described above followed by detection of the enantiomers using a mass spectrometer with a moving-belt interface [35].

5.3. Other methodologies

There are procedures for the analysis of enantiomers using nuclear magnetic resonance (NMR) and infrared (IR) detection. These techniques are typically used with the drug material itself rather than from biological samples because of the relatively large amount of sample required in these methods. For this reason, these techniques are not discussed here.

Capillary electrophoresis has recently been applied to the separation of enantiomers. Unlike chromatographic techniques, which are dependent on chiral derivatization of the compound or the use of a chiral stationary phase, this technique requires no special preparation of the sample but has been used to separate the enantiomers of ephedrine in less than 5 min [36]. Unfortunately, this technique currently lacks the sensitivity of the other techniques described, but its future looks promising.

TLC has long been used in the analysis of a wide variety of different drugs [37]. It has also been successfully used in the analysis of enantiomers of amphetamine and methamphetamine. Eskes [38] used TPC to separate the enantiomers of amphetamine and N-benzyloxycarbonyl-*l*-prolyl chloride (ZPC) to separate the enantiomers of methamphetamine. Resolution of the TPC-derivatized amphetamine enantiomers was accomplished with chloroform–methanol (197:3) and gave R_F values of 0.49 and 0.55 for the D- and L-enantiomers, respectively. The ZPC derivatives of methamphetamine enantiomers were separated using *n*-hexane–ethyl acetate–acetonitrile–diisopropyl ether (2:2:2:1) and gave R_F values of 0.61 and 0.57 for the D- and L-enantiomers, respectively. These separations were accomplished using the free base form of the drugs and it was reported that the use of the salts caused some loss of sensitivity for amphetamine and a considerable loss of sensitivity for methamphetamine under the chromatographic conditions described.

A serious limitation of TLC is the lack of quantitative results without significant effort and coupling to another technique. Given that the sample must be extracted and derivatized with a chiral derivatizing reagent, which would make the sample ready for analysis by more selective, sensitive and quantitative procedures such as GC–MS, there is little advantage of using TLC except for the relatively low cost.

6. INTERPRETATION OF ANALYTICAL RESULTS

An important adjunct to the analysis of samples is interpretation of the data. In order to properly evaluate analytical data associated with amphetamines, it is important to understand their normal metabolism. The metabolic pathway of methamphetamine is shown in Fig. 7. For

the purposes of this review, the discussion of metabolism will be directed toward amphetamine and methamphetamine enantiomers.

It has been known for many years that the enantiomers of amphetamine and methamphetamine differ in their biological activity and metabolism. The D-isomer has the greatest biological activity whereas the L-isomer is far less active [2,3,39]. Although they share some biological functions, amphetamine and methamphetamine also show activities that are unique to only one of the two enantiomers [3,40].

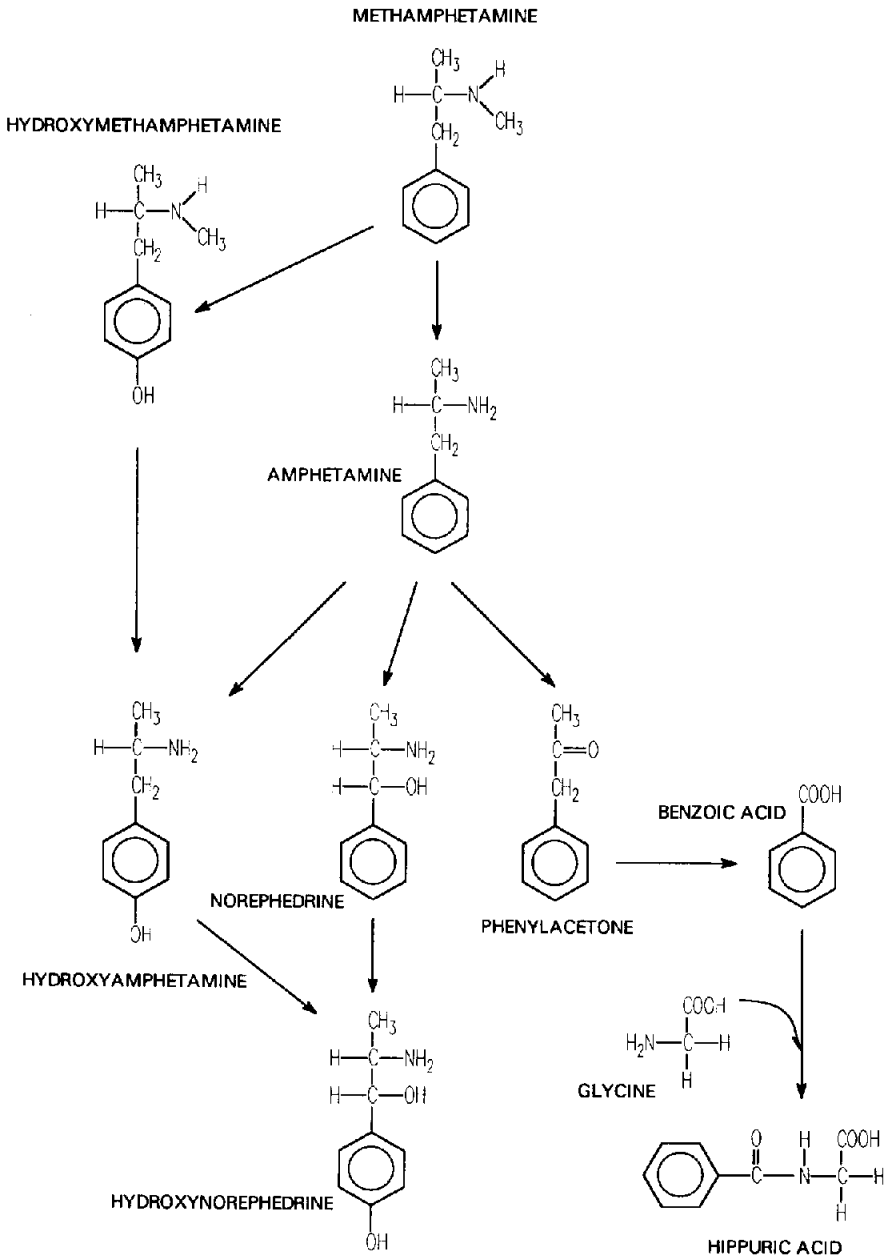


Fig. 7. Metabolic pathway for methamphetamine showing routes to various metabolic products.

6.1. Metabolic profile

The description of typical metabolic and excretion profiles for amphetamine and methamphetamine is closely related to urine pH. Whether or not the amine group is charged (protonated) has a significant impact on the retention of the compound in the body. The half-lives of amphetamine and methamphetamine range from 7 to 34 h depending on urine pH [39]. Studies that considered the metabolism of the enantiomers of amphetamine and methamphetamine have shown some significant differences in their metabolism.

After administration of racemic methamphetamine, the excretion of both enantiomers has been shown to be essentially the same for approximately the first 16 h [41]. Following this initial period, the L-enantiomer was found in higher concentration than the D-enantiomer. The reason for this appears to be the extent of metabolism of the D-enantiomer rather than actual differences in the excretion rates of the enantiomers by the kidney. pH has a significant impact on the excretion of both enantiomers, but both are equally affected by the pH. Overall excretion of the enantiomers as the intact drug is pH-dependent because the longer they are retained in the body, which is affected by pH, the more metabolism plays a role in elimination of the drugs. The longer the parent drug stays in the body, the longer the body has to metabolize it. As the metabolism of each enantiomer is different, the longer the body has to act on the D-methamphetamine the more will be metabolized, thus leaving the L enantiomer to predominate.

6.2. Source differentiation

Methamphetamine and amphetamine are available by prescription and, in such cases, their use is legitimate. Most methamphetamine and amphetamine use, however, is not legal. In determining the potential source of amphetamine or methamphetamine, the enantiomeric form of the drug can often be very helpful. This information is of particular interest with respect to methamphetamine, which is available in the L-form in an over-the-counter medication. Determination of the possibility of the amphetamine or methamphetamine actually being the metabolic product of some other compound is, in some instances, also assisted by evaluation of the enantiomeric composition. In a few circumstances this information can be used for unequivocal identification, but there are also several instances where even this information is not sufficient to identify the source. The information is in all instances, however, a powerful tool which should not be overlooked because even if unequivocal proof of the source is not possible, some potential sources can be eliminated as possibilities.

6.2.1. Methamphetamine

Methamphetamine is found in prescription form as the D-enantiomer. In the USA, it is also found as the L-enantiomer in the Vicks inhaler, which is used as a treatment for nasal congestion. It is possible for some screening procedures to give a positive result based on the presence of L-methamphetamine, but many of the immunoassay reagent systems currently available would not be expected to give a positive result because of the low cross-reactivity to this form of the drug [42–44]. In this instance, consideration must be given to the fact that a condition that would lead a person to use a Vicks inhaler would probably also involve self-administration of other over-the-counter medications that could be found at high enough concentrations to yield a positive result in a screening test. Techniques typically used for the positive identification of methamphetamine including GC–MS are not able to differentiate the enantiomer(s) present. For this reason, care must be exercised in the interpretation of data.

Illicit methamphetamine comes in two different forms. One form is essentially only D-enantiomer and the other is a racemic mixture of both enantiomers. The form produced is dependent on the precursor molecules used in the synthesis of the drug. One of the common precursors of illicit methamphetamine is L-ephedrine, and the resultant methamphetamine is essentially all

the D-enantiomer. Another common precursor is phenyl-2-propanone which, because of its symmetrical nature, yields a racemic mixture of the product methamphetamine. The illicit D-form of the drug cannot be differentiated from the prescription form because the prescribed medicinal form is also D. Examination of contaminants in the illicit drug material has been used to identify an illicit source. In biological samples, however, the concentration of these contaminants is low enough that such assays are typically not conducted. A racemic mixture of methamphetamine would indicate illicit use. The only possible exception to this would be the case where there was legitimate use of D-methamphetamine along with the concurrent use of L-methamphetamine (an extremely unlikely event) or prescription use of a racemic precursor molecule such as furfenorex.

There is some correlation between the clandestinely produced drug and the region of the world where it is found. For example, in Europe the illicit production of amphetamine far exceeds that of methamphetamine. In the USA and Asian countries, methamphetamine is the most commonly encountered. Within the USA, the east and particularly the west coast typically see D-methamphetamine produced in clandestine laboratories. In the central part of the USA, the methamphetamine is more often racemic. This region is also where most illicit amphetamine production and abuse is found in the USA [45].

The large crystalline form of methamphetamine commonly referred to as "ice" can be prepared only from D-methamphetamine. Therefore, detection of L-methamphetamine either by itself or together with the D enantiomer in a biological sample is not consistent with the use of "ice".

6.2.2. Metabolic precursors to methamphetamine

A number of different compounds have been shown to be metabolized to either amphetamine or methamphetamine. This metabolism can add significant complexity to the interpretation of analytical toxicology results. The presence of amphetamine and methamphetamine may be the result of administration of methamphetamine or the metabolism of a precursor molecule. Metabolic production of methamphetamine has been shown for benzphetamine [46–48], deprenyl [49–51], dimethylamphetamine [47,52], fencamine [53] and furfenorex [47,48].

These are the more common names encountered for these drugs, but because they are found throughout the world and in some instances are known by as many as 28 different names, a list of the chemical names and Chemical Abstracts Service (CAS) number is given in Table 1 for each of these drugs. These drugs fall into several categories, which include a monoamine oxidase inhibitor, a number of anorexics, treatment for Parkinson's disease, Alzheimer's disease, dementia, depression, prevention of stress ulcers, motion sickness, increased longevity and even sexual activity.

Interpretation of analytical data is complicated by the fact that many of these drugs which are converted by the body into methamphetamine are not excreted intact, making interpretation a far more difficult task. After administration of benzphetamine, the parent drug is excreted only for a very few hours after administration, leaving its detection unlikely [46]. Several studies of deprenyl have shown no detectable deprenyl in the urine of the user [50,54], but some have shown that it does lead to the production of a unique metabolite, desmethyldeprenyl [55,56]. Unfortunately, this metabolite is relatively short lived and detection of the amphetamines can be seen after the desmethyldeprenyl is completely eliminated.

In addition to the parent compound, if it is present, the enantiomeric composition of the amphetamine and methamphetamine can be a useful tool to differentiate the use of some of these precursor drugs from direct use of amphetamine and methamphetamine. One example of this is deprenyl, which is the L-form. The product methamphetamine and amphetamine are therefore also in the L-form [54]. Detection of any D-methamphetamine or D-amphetamine from an individual would indicate that the drug was not the product of deprenyl administration.

This use of enantiomeric data is very useful but often cannot solely determine the source of the

TABLE I

COMMON NAMES, CHEMICAL ABSTRACTS SERVICE NUMBERS AND SYSTEMATIC NAMES OF COMPOUNDS METABOLIZED TO METHAMPHETAMINE

Drug name	CAS No.[a]	Systematic name
Benzphetamine	156-08-1	N,α-Dimethyl-N-(phenylmethyl)benzeneethanamine
Deprenyl	(*R*)-14611-51-9 2323-36-6	N,α-Dimethyl-N-2-propynylbenzeneethanamine
Dimethylamphetamine	075-96-1	N,N-α-Trimethylbenzeneethanamine
Fencamine	28947-50-4	N-Methyl-N-(1-methyl-2-phenylethyl)-N'-3,7-dihydro-1,3,7-trimethyl-8-({2-[methyl-(1-methyl-2-phenylethyl)amino]ethyl}-amino)-1*H*-purine-2,6-dione
Furfenorex	(±)-13445-60-8 (+)-3776-93-0	N-Methyl-N-(1-methyl-2-phenylethyl)-2-furanmethanamine

[a] The Chemical Abstracts Service numbers refer to the compound itself. Other numbers may also apply to the drug in some other formulation (*e.g.*, 14611-51-9 is the CAS number for deprenyl and 14611-52-0 is the number for deprenyl hydrochloride). Only the numbers for the drug itself are listed.

drug. It can, however, be a powerful adjunct when combined with other data. Certainly if there is a valid medical prescription for the drug and the enantiomeric composition found is consistent, the likelihood is that there was not direct use of either amphetamine or methamphetamine.

7. CONCLUSIONS

Analysis of methamphetamine enantiomers by a number of alternative procedures is well established. In the evaluation of the effects of methamphetamine on the body and mechanisms of action, it is important to consider the use of these techniques. In the forensic area, it is extremely important to ensure that the identification of the enantiomer present is accomplished. The potential misinterpretation of data due to the lack of information concerning the distribution of enantiomers is far too great to ignore. With the use of readily available analytical procedures, the composition can be proved and the significance of the presence of these compounds can be evaluated.

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