Derivatization

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Abstract

Gas chromatography-mass spectrometry (GC-MS) is still one of the most widely used analytical techniques in the forensic toxicology laboratory. Chemical derivatization is used to enhance the volatility, temperature stability, and detectability of drugs. It is an unavoidable requirement for some drugs and metabolites, particularly those with polar functional groups. Although chemical derivatization is an additional sample preparation step, chromatographic characteristics, stability, and overall improvements in detectability and specificity can be achieved. The most common derivatization techniques include silylation, acylation, and alkylation. A wide variety of derivatization reagents can be employed for this purpose. Approaches to derivatization are discussed for drugs of forensic interest, including amphetamines, benzodiazepines, cocaine metabolites, and opiates.

Keywords

Chemical derivatization · Gas chromatography · Silylation · Acylation · Alkylation · Chiral separation

Abbreviations

BF_3	Boron trifluoride
BSA	*N,O*-bis(trimethylsilyl) acetamide
BSTFA	*O,N*-bis(trimethylsilyl) fluoroacetamide
HFBA or HFAA	Heptafluorobutyric acid anhydride
HFBI	Heptafluorobutyrylimidazole
HFIP	1,1,1,3,3,3-hexafluoroisopropanol
l-TPC	*l*-*N*-trifluoracetyl-prolyl chloride
MBTFA	*N*-methyltrifluoroacetamide
MSTFA	*N*-methyl-*N*-trimethylsilyltrifluoroacetamide
MTBSTFA	*N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide
MTPA	(*R*)-(-)methoxytrifluoromethylphenylacetic acid
PFBBr	Pentafluorobenzyl bromide

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PFPA or PFAA	Pentafluoropropionic acid anhydride
PFPI	Pentafluoropropany limidazole
PFPOH	2,2,3,3,3-Pentafluoro-1-propanol
TBDMDMCS	Tert-butyldimethylchlorosilane
TFAA	Trifluoroacetic acid anhydride
TFAI	Trifluoroacetylimidazole
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
TMSI	Trimethylsilylimidazole

Introduction

Forensic toxicologists are responsible for the identification of drugs and metabolites in biological fluids. Gas chromatography-mass spectrometry remains one of the most universally used and robust techniques in forensic toxicology. Gas chromatographic separation is particularly effective for compounds that are non-polar, thermally stable, and volatile. It has been used for the detection, identification, and quantitation of a wide range of xenobiotics. However, the presence of polar functional groups, or the introduction of such groups during biotransformation can be problematic. Under these circumstances, drugs or metabolites may exhibit poor chromatographic properties. Modification of the chemical structure of the molecule during the derivatization process can improve both separation and detection. The most frequently used derivatization techniques for GC are reviewed, including silylation, acylation, and alkylation.

Derivatization for GC-MS

If a molecule is too polar, non-volatile, thermally liable, produces insufficient diagnostic ions, or has poor chromatographic properties (e.g., peak tailing or poor separation), derivatization may be necessary. Derivatization involves the chemical modification of an existing drug or metabolite to produce a new compound, with enhanced chromatographic properties. Additionally, since the chemical structure is changed, the drug may fragment differently, potentially improving the abundance, or the diagnostic value of the resulting ions. Advantages of derivatization as it relates to gas chromatography include:

- Decreased polarity
- Increased molecular mass
- Improved detectability
- Increased specificity
- Increased volatility
- Increased thermal stability

Decreasing the polarity of a molecule is beneficial because it results in less adsorption onto the stationary phase, which can improve peak shape and enhance resolution. Derivatization, which increases the molecular mass, can also improve the specificity of ions, which is particularly important if selected ion monitoring (SIM) is being used. Some drugs of interest produce poorly diagnostic mass spectra when analyzed directly by GC-MS. For example, during electron impact (EI) ionization, methamphetamine readily fragments to produce the tropylium ion base peak at *m/z* 91 ($C_7H_7^+$) and the $C_3H_8N^+$ ion (*m/z* 58) from the phenethylamine side chain, neither of which are highly specific (Fig. 12.1). Smaller fragments are less diagnostic because of their increased abundance in nature. Therefore, the absence of the molecular ion can be a disadvantage, particularly for drugs with small molecular masses. Therefore, chemical derivatization can not only increase the molecular mass, but improve the mass spectral characteristics of the drug, yielding more specific ions with greater diagnostic value. This is depicted in Fig. 12.1, which shows EI mass spectra for underivatized and derivatized methamphetamine.

Derivatization typically involves the replacement of an active hydrogen on various functional groups. Target functional groups can include amines, alcohols, phenols, ketones, carboxylic acids, and others. The general reaction for derivatization is as follows:

$$R_1 - AH + R_2 - D \rightarrow R_1 - AD + R_2 - H$$

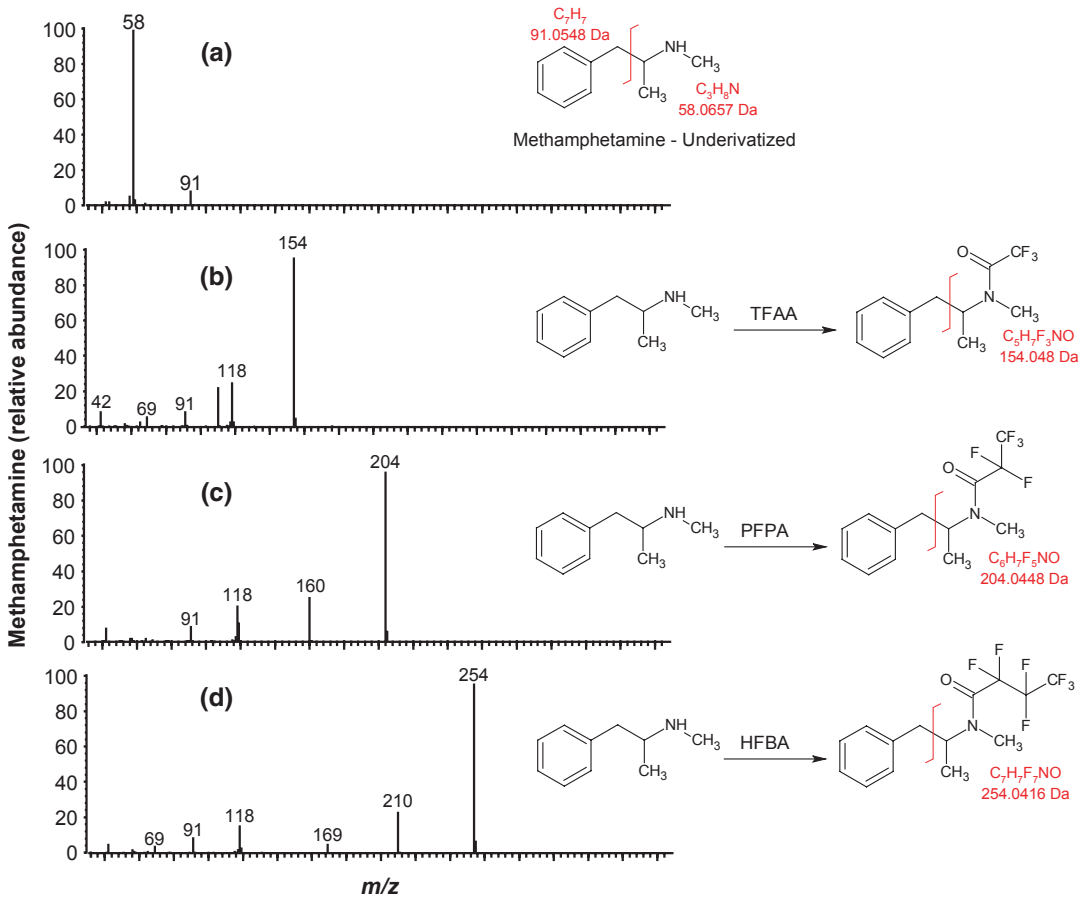

Fig. 12.1 Mass spectra of methamphetamine underivatized (**a**) and derivatized with TFAA (**b**), PFPA (**c**), and HFBA (**d**)

where "A" is an oxygen, sulfur, nitrogen, or similar atoms found in functional groups, "H" is an active hydrogen, and "D" is the functional group on the derivatization agent. There are multiple derivatization agents and a single drug may be derivatized using a variety of different approaches. Abbreviations for the most common derivatization reagents can be found at the end of the chapter. The ideal derivatization technique for a certain molecule or drug should:

- Produce a single, stable, reproducible, and high yield derivative
- Result in simple and fast reaction using straightforward laboratory techniques
- Achieve the desired modification and chemical properties
- Use non-hazardous reagents
- Not result in detector fouling

There is potential to produce more than one derivative if the molecule contains multiple functional groups. Side products may be produced during the reaction, which can change the chemical environment of the reaction (e.g., make it more acidic) and compromise the stability of the derivative product. A disadvantage of derivatization is the increased sample preparation time in the laboratory; additional post-derivatization clean-up may also be required to remove excess reagent and/or by-products prior to analysis. Failure to remove these prior to injection can result in deterioration of the stationary phase, detector fouling and increased instrument maintenance.

Once polar functional groups within the molecule have been identified, the appropriate derivatization technique can be selected.

Silylation

Silylation is one of the most universal derivatization techniques as it is applicable to numerous functional groups, including alcohols, phenols, amines, thiols, and carboxylic acids (Table 12.1). Silylation involves the introduction of a silyl moiety through substitution at the active hydrogen.

The advantages of silylation include:

- Applicability to a wide range of functional groups
- Variety of reagents commercially available
- Ease of preparation
- Thermally stable derivatives
- Excellent chromatographic characteristics
- Increased volatility
- Amenable to direct GC analysis (no clean-up)

Trimethylsilyl (TMS) are among the most common derivatives utilized, although many other alkylated silyl groups can be used. Common derivatization reagents for silylation are listed in Table 12.1. The replacement of an active hydrogen with TMS [R–Si$(CH_3)_3$] increases the molecular weight of the drug by 72 Da. In EI spectra the *m/z* 73 often features prominently and is not considered a diagnostic ion because it is not associated with the molecular structure of the drug itself. Higher alkyl homologs further increase molecular weight (e.g., *t*-butyldimethylsilyl or *t*-BDMS). They also have increased hydrolytic stability and improved mass spectral characteristics. Their EI mass spectra are often characterized by abundant $[M-57]^+$ ions due to the loss of the tert-butyl group. Despite this advantage, these bulky groups present difficulties for sterically hindered groups on some drug molecules.

While silylation is applicable to various functional groups, alcohols (primary > secondary > tertiary) are the most reactive with silyl reagents, followed by phenols, carboxylic acids, and amines (primary > secondary). *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and

Table 12.1 Derivatization of common functional groups

Functional group	Derivatization	Reagent	Derivatives
Alcohols/ phenols	Silylation	BSTFA, BSA, HDMS, MSTFA, TMSI, TMCS (catalyst)	Trimethylsilyl (TMS)
		MTBSTFA, TMCS (catalyst)	*t*-Butyldimethylsilyl (*t*-BDMS)
	Acylation	TFAA, TFAI, MBTFA	Trifluorobutyramides
		PFPA/PFAA	Pentafluorobutyramides
		HFAA/HFBA, HFBI	Heptafluorobutyramides
	Alkylation	PFBBr	Pentafluorobenzyl ethers
Amines	Silylation	BSTFA, BSA, MSTFA, TMCS (catalyst)	Trimethylsilyl (TMS)
		MTBSTFA, TBDMCS (catalyst)	*t*-Butyldimethylsilyl (*t*-BDMS)
	Acylation	TFAA, MBTFA	Trifluorobutyramides
		PFPA/PFAA	Pentafluorobutyramides
		HFAA/HFBA	Heptafluorobutyramides
Carboxylic acids	Silylation	BSTFA, BSA, MSTFA, TMSI, TMCS (catalyst)	Trimethylsilyl (TMS)
		MTBSTFA, TMCS (catalyst)	*t*-Butyldimethylsilyl (*t*-BDMS)
	Alkylation	PFBBr	Pentafluorobenzyl ethers
		BF_3/Methanol	Methyl esters

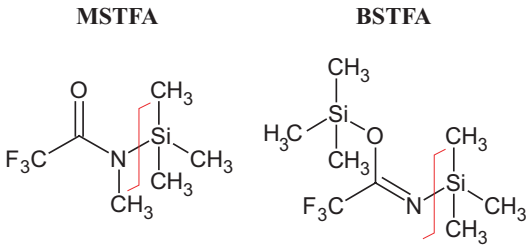

Fig. 12.2 Chemical structures of commonly used silylation reagents MSTFA and BSTFA with severed bond indicating the TMS group

N,O-bis(trimethylsilyl)fluoroacetamide (BSTFA) are widely utilized for this purpose (Fig. 12.2). Other silylation reagents include trimethylsilylimidazole (TMSI) and *N,O*-bis(trimethylsilyl) acetamide (BSA) (Table 12.1). While many of these reagents have similar silylation potencies, increased volatility of the reagent can be beneficial because it can reduce interfering peaks in the chromatogram. Silylation reactions occur through nucleophilic attack, so their efficiency depends on good leaving groups. For this reason, BSTFA is a more effective silylating reagent than BSA, because trifluoroacetamide is a better leaving group, reacting faster and more completely than BSA for most compounds.

Catalysts are often employed during silylation, particularly if sterically hindered functional groups (e.g., tertiary alcohols or steroids) are the target molecule. The most commonly used catalyst for silylation is trimethylchlorosilane (TMCS). A commonly used combination is BSTFA + 1% TMCS. Alternatively, some mixtures of silylating reagents and catalyst are used (e.g., BSA/TMSI/TMCS (1:1:1, v/v/v)). TMSI is a particularly useful derivatization reagent because it is not reactive toward amines and therefore has greater selectivity than BSTFA and MSTFA. If the drug of interest contains both hydroxyl and amine groups, TMSI could be advantageous in terms of the production of a single derivative. The use of TMSI will also preserve keto groups and prevent the formation of enols during the reaction.

It is very important when performing silylation reactions to use aprotic organic solvents, as these reagents are easily hydrolyzed when exposed to aqueous conditions and will react with any active hydrogens. GC column stationary phase should not contain any active sites as well. Pyridine is sometimes used as the solvent because it is an acid scavenger and helps drive the reaction forward. However, it can also result in peak tailing. Silylation reactions can be performed in the absence of solvent if the compounds of interest are sufficiently soluble in the reagent. Finally, silylating reagents are typically moisture sensitive, as are the derivatives themselves.

Although the reaction requires heat, most are effective at moderate temperatures (e.g., 60–80 °C), and on-column derivatization is also possible for some silylating reagents. During method development, derivatization conditions should be carefully evaluated and optimized to ensure that the reaction is either complete, or reproducible. If derivatization does not go to completion, it is important to ensure that the internal standard selected behaves in an identical fashion to the drug or interest. This can be achieved using isotopically labeled internal standards. If compounds of interest undergo derivatization, it is not acceptable to use an internal standard that does not derivatize. The completeness and reproducibility of the derivatization are particularly important when drugs contain more than one derivatization site (e.g., morphine). The trimethylsilyation of morphine is shown in Fig. 12.3, whereby both OH groups are derivatized, resulting in a di-TMS derivative.

Forensically relevant analytes that are frequently derivatized using silylation reactions include cannabinoids (e.g., Δ^9-THC, carboxy-THC, hydroxy-THC), cocaine metabolites, opiates, and benzodiazepines. Their reactivity toward so many functional groups, ease of preparation, and limited sample clean-up prior to GC injection makes silylation a popular choice in forensic toxicology applications. Advantages and disadvantages of the most common derivatization approaches are summarized in Table 12.2.

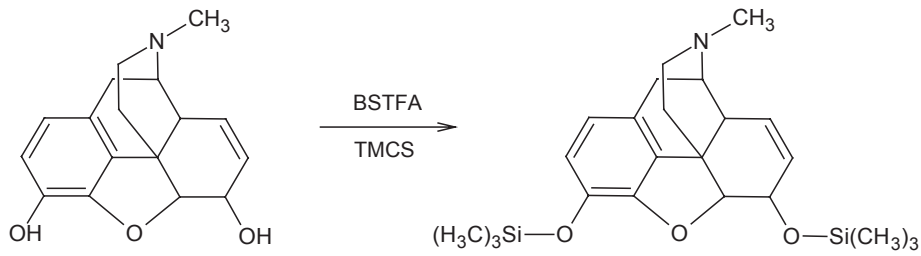

Fig. 12.3 Silylation of morphine using BSTFA + 1% TMCS

Table 12.2 Advantages and disadvantages of derivatization approaches

Derivatization type	Advantages	Disadvantages
Silylation	• Large number of silylating reagents available • Easy to prepare • No additional clean-up necessary • Reactive towards many functional groups (-CO_2H, NH_2, OH, Amide) • Useful when derivatizing drugs with more than one functional group type (e.g., –OH and –CO_2H, carboxy-THC)	• Must use aprotic solvents • Reagents and derivatives are moisture sensitive • Required anhydrous conditions • Potential for multiple derivatives
Acylation	• Large number of reagents available • Fluorinated derivatives with increased molecular weight and longer retention times • Derivative hydrolytically stable • Addition of halogenated carbons can increase detectability	• Acid by-products for some reagents require removal prior to GC analysis • Acylation reagents moisture sensitive • Reagents hazardous and odorous • Not reactive toward carboxylic acids
Alkylation	• Reaction conditions vary from strongly basic to strongly acidic • Some reactions possible in aqueous solution • Derivatives generally stable	• Can be used in conjunction with acylation and silylation • Reagents often toxic • Conditions may be harsh

Acylation

Another common derivatization technique is acylation, which involves the introduction of an acyl moiety (RCO–) onto a molecule through substitution of an active hydrogen. Acylation is an ideal technique for polar molecules containing hydroxyls, thiols, or amines and converts them into esters, thioesters, and amides (Fig. 12.4). The advantage of using acylation as a derivatization technique is the formation of stable derivatives that are highly volatile and have increased sensitivity with characteristic mass spectral fragmentation due to the increase in molecular weight.

Acyl derivatives can be formed using three different types of reagents: acyl halides, acid anhydrides, or reactive acyl derivatives, such as acylated imidazoles. Acetic anhydrides, particularly perfluoroacyl anhydrides, are among the most common reagents for acylation. Examples of these reagents include trifluoroacetic acid anhydride (TFAA), pentafluoropropionic acid anhydride (PFPA or PFAA), and heptafluorobutyric acid anhydride (HFBA or HFAA) (Fig. 12.5). The fluorinated acyl groups increase molecular weight and retention time significantly. This electronegativity can be further exploited if negative chemical ionization is to be used for

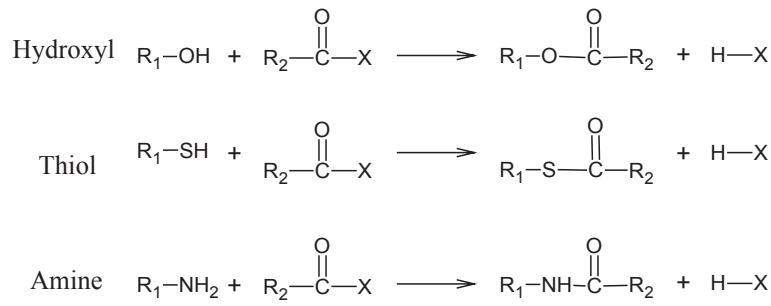

Fig. 12.4 General reaction scheme for acylation of hydroxyls, thiols, and amines

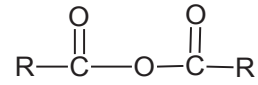

Fig. 12.5 General structure of perfluoroacyl acid anhydrides

Reagent	R	Additional Molecular Weight
TFAA	$O = C - CF_3$	210
PFAA	$O = C - CF_2 - CF_3$	310
HFAA	$O = C - CF_2 - CF_2 - CF_3$	410

analytes that are present at low concentration (e.g., triazolam) or if electron capture detection (ECD) is used.

While acyl halides and acid anhydride reagents produce stable and volatile acyl derivatives, they form acid by-products that must be removed prior to GC analysis. These reactions may be performed in pyridine, tetrahydrofuran, or other solvents capable of accepting the acid by-product. Even if a basic acceptor is used to neutralize them, additional steps are often necessary to remove excess derivative prior to analysis. Their removal is relatively straight-forward using simple evaporation (under nitrogen) or liquid-liquid extraction, but it does require an additional sample preparation step.

Additional common reagents include *N*-methyltrifluoroacetamide (MBTFA) and perfluoro-acylimidazoles, such as trifluoroacetylimidazole (TFAI), pentafluoropropanylimidazole (PFPI), and heptafluorobutyrylimidazole (HFBI). These reagents also produce highly volatile derivatives, but do not produce by-products that would need to be removed prior to analysis by GC; the by-products are inert and highly volatile. Caution is needed when using these reagents however, because they are sensitive to aqueous environments.

Acylation has been widely utilized for the derivatization of many drug classes, notably amphetamines and opiates. Since they are not reactive toward carboxylic acid groups, metabolites of cocaine and THC require an additional derivatization step (discussed later). LSD has been derivatized using trifluoroacetylimidazoles. MBTFA has been used to produce carbohydrate derivatives. Acetic anhydride can also be used for the acylation of alcohols, phenols, and amines. Although the resulting esters are more stable than silylated derivatives, the molecular weight increase is minimal.

Alkylation

While not as common as silylation or acylation, alkylation is another derivatization technique used to improve detection and chromatographic

characteristics of drugs by GC analysis. Alkylation involves the substitution of an alkyl or aryl group at an active hydrogen on hydroxyls, carboxylic acids, thiols, and amines to produce ethers, esters, thioethers, thioesters, *N*-alkyl amines, and *N*-alkyl amides. It is particularly widely used for the modification of acidic hydrogens on carboxylic acids and phenols. The advantages of alkylation are the formation of stable derivatives that have higher volatility than the original molecule. However, as the acidity of the active hydrogen decreases, stronger alkylating reagents are required.

Reagents include substituted benzyl bromides, unsubstituted benzyl bromides, and alkyl halides such as aliphatic bromides and iodides. Common reagents include boron trifluoride (BF_3)/methanol and pentafluorobenzyl bromide (PFBBr).

Alkylation of carboxylic acids is also possible by esterification with alcohols. Drugs or metabolites containing a carboxylic acid can be esterified using 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) or 2,2,3,3,3-pentafluoropropanol (PFPOH). These reagents convert carboxylic acids into the corresponding fluorinated ester. This approach is often used in combination with other derivatization reagents. For example, acylation and esterification of -OH and $–CO_2H$ using both PFPA and PFPOH when dealing with polyfunctional compounds (e.g., benzoylecgonine). Although silylation using a single reagent can be used to derivatize carboxylic acids and phenols, these derivatives are less stable than their alkylated counterparts.

Other Derivatization Techniques

Oximes

Some molecules contain more than one functional group; however, only one functional group may need to be transformed during derivatization reaction. This often occurs when the ketone group of a compound does not need to undergo derivatization and another more polar functional group does. An example is the silylation of the hydroxyl group on steroids. Oximes can be used to prevent the keto group from interfering with the derivatization of another functional group, essentially protecting it. Using this approach, a pentafluorobenzyl oxime can be prepared using pentafluorobenzyloxylamine. Oxime formation has been widely reported for steroids, and also for the keto-opioids (discussed later).

Cyclization

Another derivatization technique involving polyfunctional group molecules is cyclization, where there is a simultaneous reaction with two proximal active functional groups to form a cyclic derivative. In order for a cyclic derivatization to occur, there must be the appropriate amount of spatial separation between the two functional groups and the ring must be stable. Reagents using cyclization include boronic acids [$R–B(OH)_2$] in the presence of aprotic, organic solvents such as pyridine or acetone.

The advantage of cyclization over other derivatization techniques is the potential to produce one derivative versus multiple derivatives. However, multiple derivative products could be formed if the molecule has more than two functional groups with an active hydrogen.

Chiral Derivatization

Separation of chiral compounds (e.g., *d/l* methamphetamine) presents a unique challenge using gas chromatography. If derivatization is to be avoided, stereoisomers can be separated using a chiral stationary phase. However, an alternative approach is to derivatize the molecule using an optically pure reagent, followed by separation on a traditional (achiral) chromatographic phase.

Chiral separation on GC has been achieved for multiple analytes, including methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), methadone, and a variety of anti-inflammatory medications. Fluroacyl-prolyl chlorides are commonly used: heptafluorobutyryl

prolyl chloride and *l*-*N*-trifluoracetyl-prolyl chloride (*l*-TPC) have been used to separate the two methamphetamine enantiomers and *S*-(-)-trifluoroacetyl prolyl chloride has been used to separate MDMA chiral forms. Additional reagents include (*R*)-(-)methoxytrifluoromethylphenylacetic acid (MTPA) for methamphetamine, (-)methyl chloroformate for methadone, and *S*-(-)-1-(-1-naphtyl)ethylamine for anti-inflammatory drugs.

Although chiral derivatization is more specialized and requires optically active and pure reagents of sufficient volatility, it avoids the costs associated with a chiral stationary phase.

Derivatization by Drug Class

Amphetamines

Amphetamines are frequently encountered in forensic toxicology casework. Amphetamines and structurally related analogs (e.g., cathinones) contain a primary, secondary or tertiary amine. Active hydrogens on primary and secondary amines benefit from chemical derivatization. The EI mass spectra of amphetamine and methamphetamine are each dominated by one poorly diagnostic ion, *m/z* 44 and *m/z* 58, respectively. The derivatization of these molecules improves their chromatographic and mass spectral properties. Derivatization also allows for these relatively small molecules to be better separated from structurally related endogenous compounds found in biological matrices.

Although primary and secondary amines readily undergo silylation and acylation, the latter is the most common approach. Acylation using HFBA, PFPA, or TFAA, and silylation using MTBSTFA have been reported (Fig. 12.6). Other techniques including the use of propylchloroformate, pentafluorooctanoyl chloride, and combination of acid anhydrides with organic solvents are also possible. Amphetamines bearing tertiary amines, including the pyrrolidine-type cathinones (e.g., methylenedioxypyrovalerone (MDPV), α-pyrrolidonopentiophenone (α-PVP)) do not derivatize with these techniques due to the absence of an active hydrogen. Chiral separation of amphetamines by GC is commonly achieved using the chiral derivative, *l*-TPC described earlier. A schematic for this reaction is shown in Fig. 12.7.

Benzodiazepines

While derivatization is not necessary for the analysis of most benzodiazepines by GC-MS, it can be used to improve chromatographic characteristics and improve volatility of the more polar benzodiazepines, such as oxazepam,

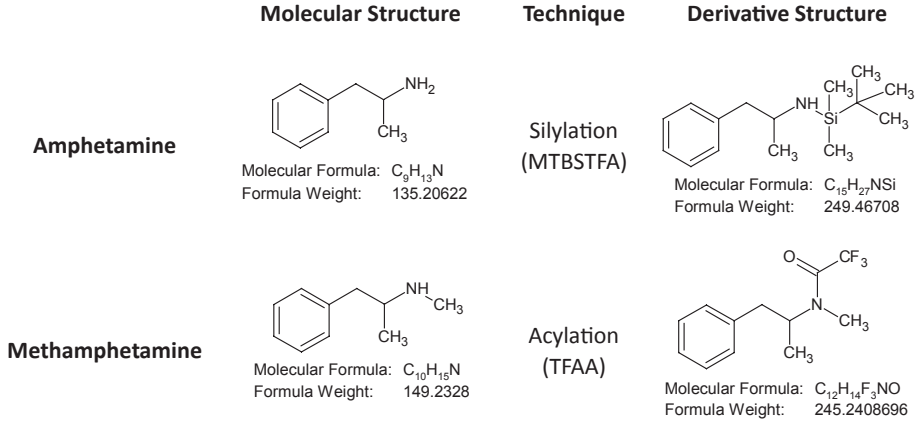

Fig. 12.6 Derivatization of amphetamine and methamphetamine

temazepam, and lorazepam (containing a hydroxyl group) or clonazepam (containing a nitro group). Although 1,4-benzodiazepines are most common, the drug class is extensively functionalized at many sites on the molecule. Considering the wide variety of benzodiazepine structures, derivatization has been achieved using an assortment of techniques, including silylation, acylation, and alkylation. Silylation remains one of the most popular approaches for the identification of benzodiazepines and their metabolites (Fig. 12.8).

Fig. 12.7 Chiral derivatization of methamphetamine using *l*-TPC

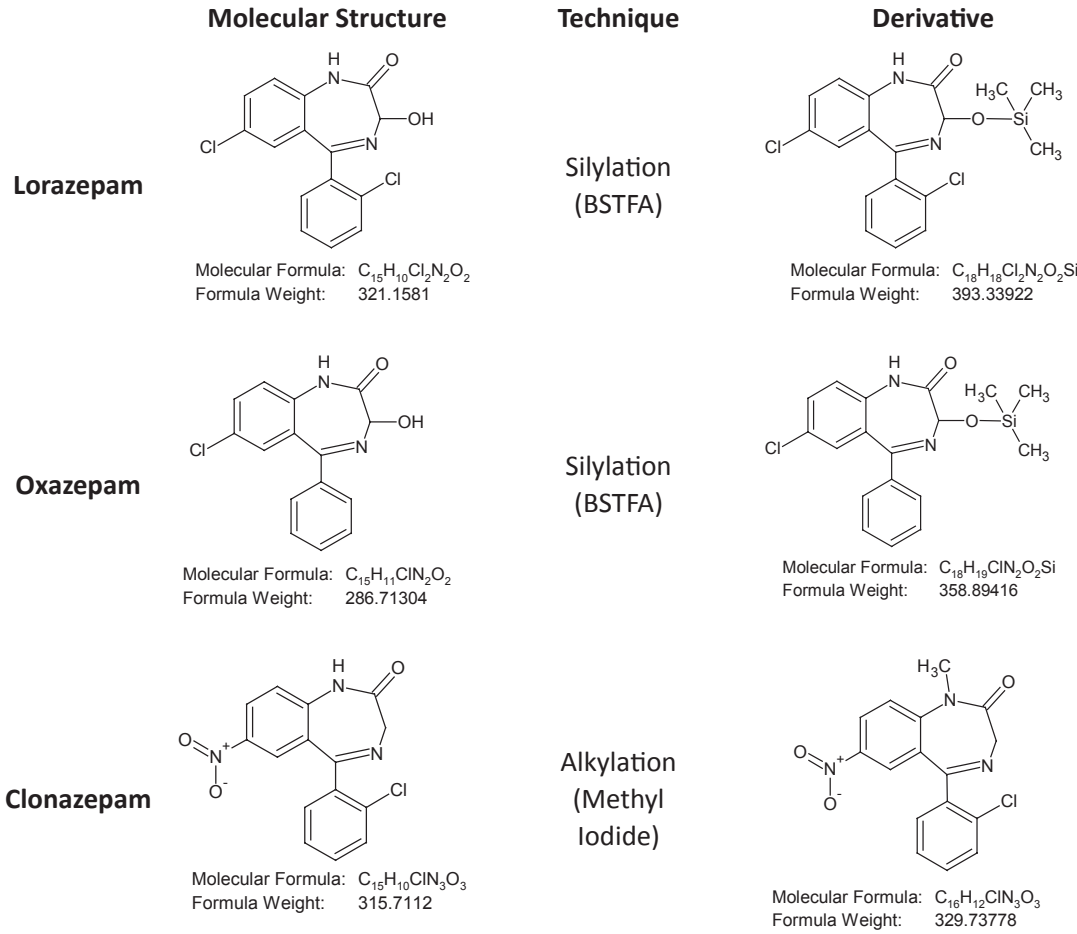

Fig. 12.8 Derivatization of benzodiazepines by silylation and alkylation

Cannabinoids

Derivatization is necessary for the identification of THC and its metabolites in biological samples. While acylation of the hydroxy (e.g., PFPA) and esterification of the carboxylic acid (e.g., HFIP) have been reported, silylation is more convenient because it derivatizes both functional groups. Commonly used silylation reagents include BSTFA, MSTFA, and MTBSTFA (Fig. 12.9).

Opioids

Morphine and related compounds often contain multiple sites for derivatization. Silylation and acylation are the most commonly used techniques. Common reagents used for silylation of these analytes include BSTFA or MSTFA, often in the presence of a catalyst (TMCS) (Fig. 12.10).

Structurally similar to opiates, semi-synthetic opioids also require derivatization prior to analysis by GC-MS. The semi-synthetic opioids (oxycodone, oxymorphone, hydrocodone) include a keto-substituent, which decreases the volatility of these analytes. Derivatization can be achieved using silylation or acylation; however, both the enol and keto derivative may form. Protection of the ketone group by the formation of an oxime is possible. For keto-opioids this is commonly achieved using hydroxylamine or methoxylamine. This is depicted in Fig. 12.11 using hydrocodone.

Cocaine

Chromatographic properties of cocaine metabolites (particularly benzoylecgonine) are significantly improved by derivatization. This can be

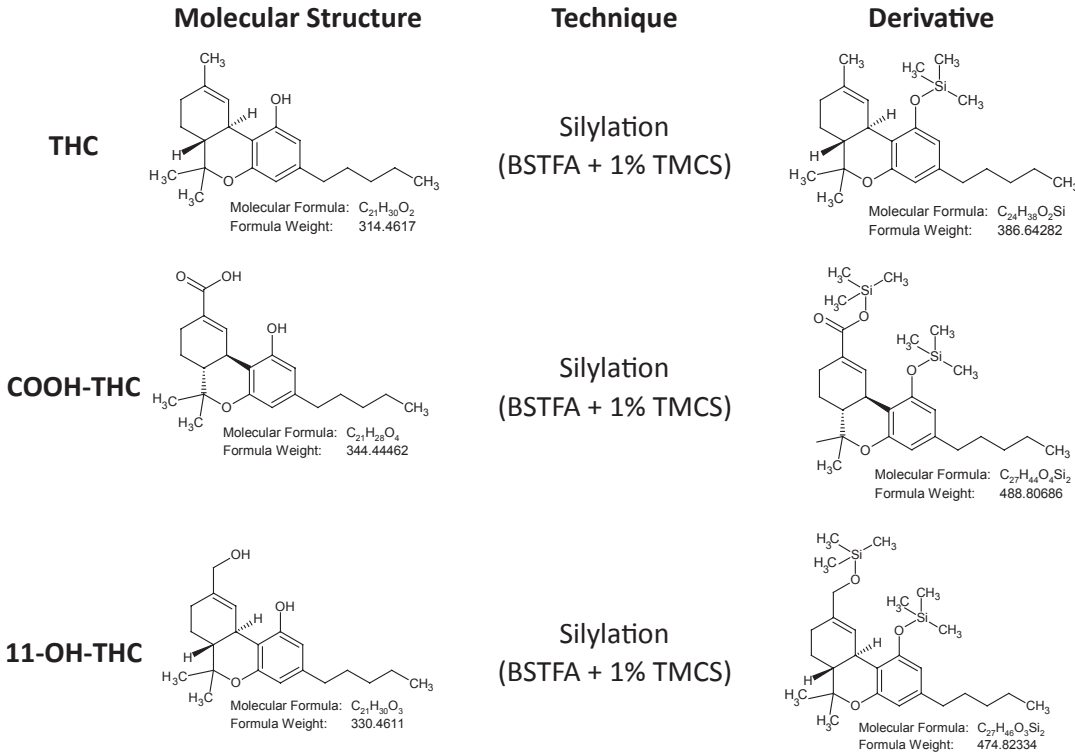

Fig. 12.9 Derivatization of cannabinoids by silylation

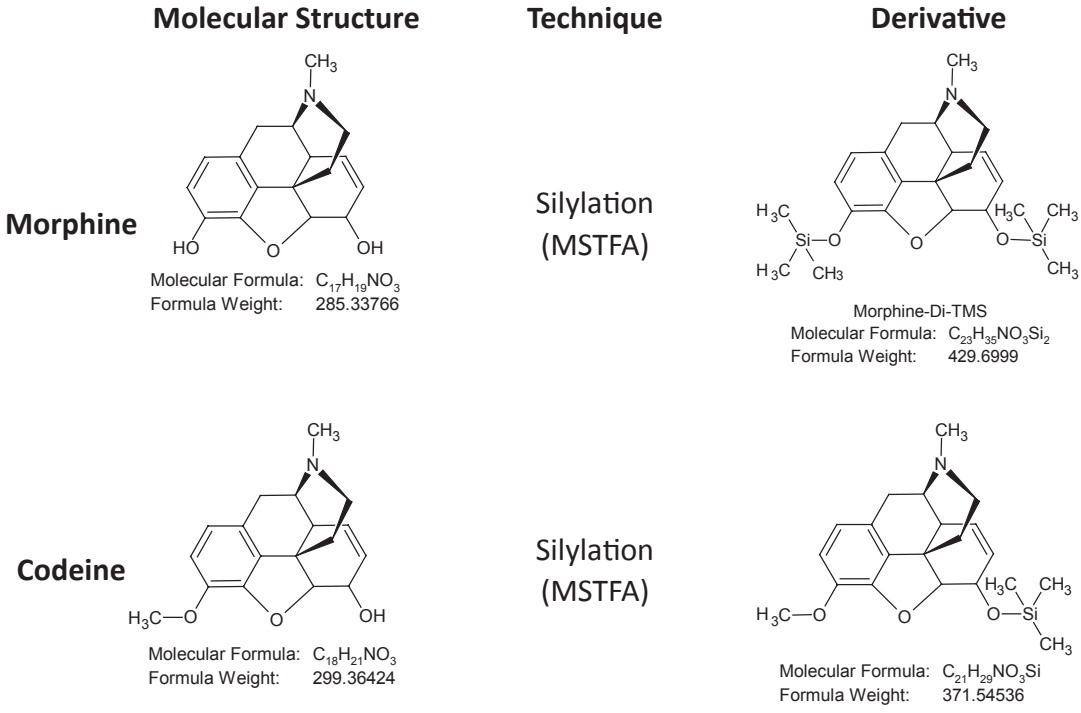

Fig. 12.10 Derivatization of opiates by silylation

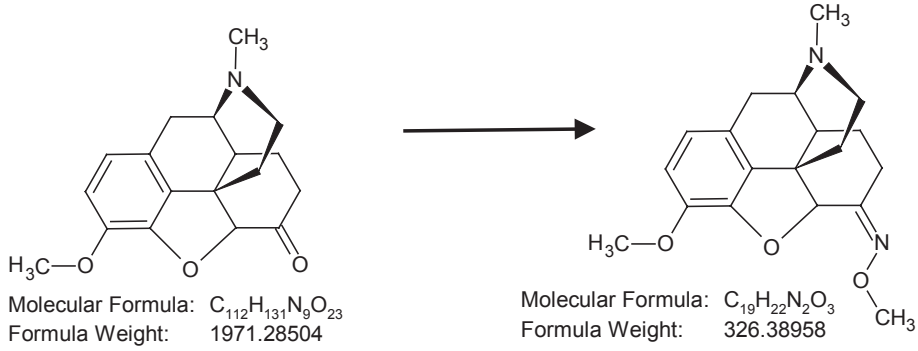

Fig. 12.11 Derivatization of select opioids

achieved using silylation, which is reactive towards both –OH and –CO_2H, or by esterification. Higher abundances of BE have been reported using acylation when compared to silylation. As described earlier, esterification requires the use of two derivatization reagents. A schematic for the esterification of cocaine metabolites using PFPA/PFPOH is shown in Fig. 12.12.

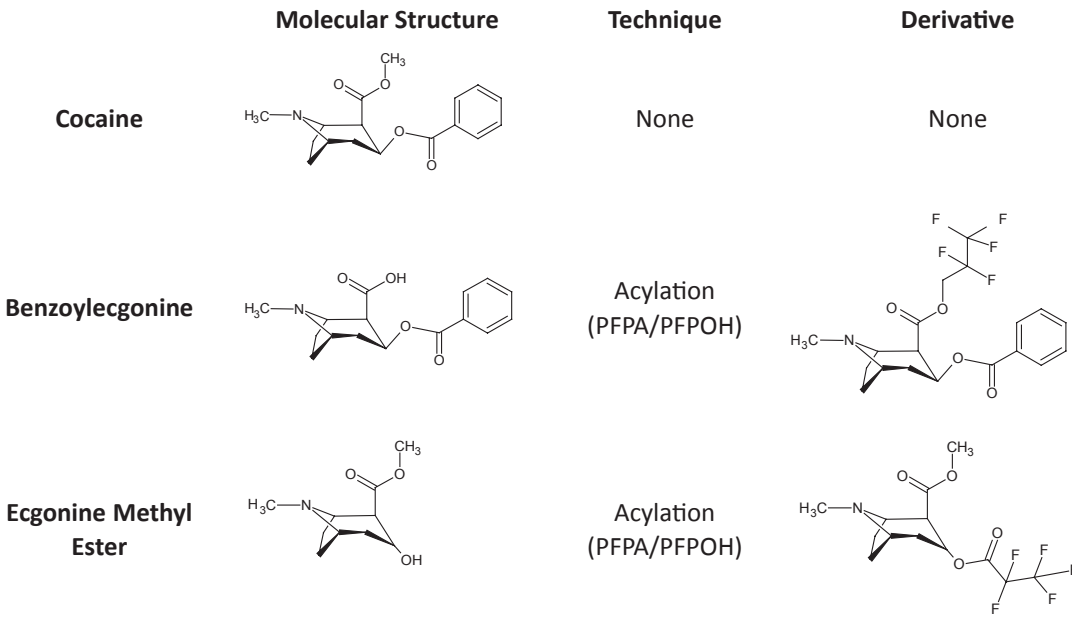

Fig. 12.12 Derivatization of cocaine and metabolites

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