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Review Article

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Chemical Derivatization for the Analysis of Drugs by GC-MS — A Conceptual Review

DONG-LIANG LIN[1], SHENG-MENG WANG[2], CHIH-HUNG WU[3], BUD-GEN CHEN[3] AND RAY H. LIU[3]*

[1.] Department of Forensic Toxicology, Institute of Forensic Medicine, Ministry of Justice, Taipei City 106, Taiwan (R.O.C.)

[2.] Department of Forensic Sciences, Central Police University, Kuei-Shan Hsiang, Taoyuan County 333, Taiwan (R.O.C.)

[3.] Department of Medical Technology, Fooyin University, 151 Ching-Hsueh Rd., Tao-Liao Township, Kaohsiung County 831, Taiwan (R.O.C.)

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ABSTRACT

Drugs are often chemically derivatized prior to their GC-MS analysis for the following reasons: (a) to bring the analytes to the chemical forms that are more compatible to the chromatographic environment; (b) to create a separation mechanism or to maximize resolution efficiency; (c) to improve detection or structural elucidation effectiveness; or (d) to make use of the analytes' specific structural features for analyticl needs. Analytes that are strongly acidic, basic or with functional groups, that may not vaporize or may interact with (irreversibly or reversibly) silanol groups or contaminating compounds present in the chromatographic system, can be more effectively analyzed after chemical derivatization. Enantiomers can be chromatographically resolved by achiral columns after being converted into diastereomers using chiral reagents; derivatization may also bring the retention time of the targeted analytes to a more desirable range. Introduction of certain elements or groups through chemical derivatization may enhance the detector's response or generate mass spectra helpful to the elucidation of the analytes' structural features. In conclusion, commonly used derivatization reagents for silylation, acylation, and alkylation are summarized along with comments on some practical considerations.

Key words: chemical derivatization, enantiomers, silylation, acylation, alkylation

INTRODUCTION

Ideally, an analyte should be tested in its original form. The conversion of an analyte to a different form (derivative) prior to its analysis involves an additional chemical step that may add cost. It may also complicate the interpretation of the analytical data because the derivatization reaction may introduce impurities, uncertainty on the completeness of the analytes' conversion, and other interference factors. However, for the reasons listed below, drugs are often derivatized prior to their gas chromatographic (GC) analysis[(1)]:

1. Conferment of volatility;
2. Improvement of stability;
3. Improvement of chromatographic properties;
4. Improvement of separations;
5. Functional group analysis;
6. Provision for selective detection (non-mass spectrometric);
7. Production of mass shift in mass spectra;
8. Modification of fragmentation; and
9. Use of derivatives in conjunction with chemical ionization.

* Author for correspondence.
Tel: +886-9-3636-3732; Fax: +886-7-782-7162;
E-mail: mt124@mail.fy.edu.tw, rayliu@uab.edu

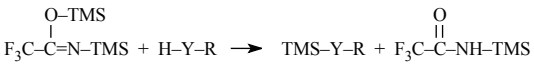
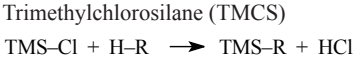
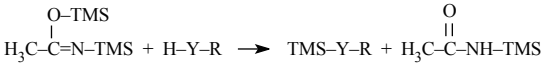
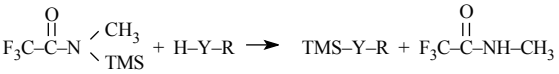
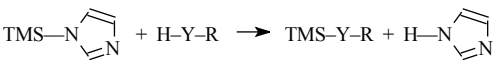
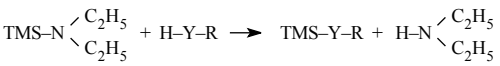
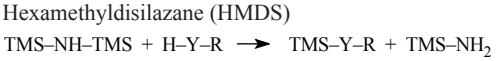
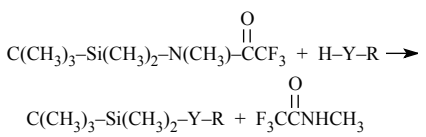
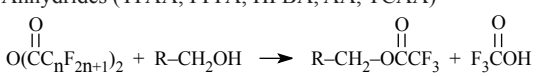
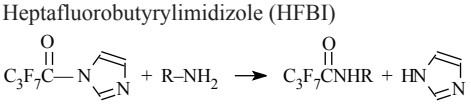
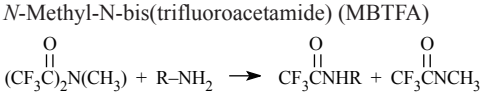
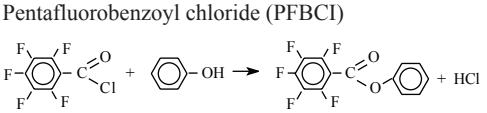
These reasons can be grouped into the following categories: bringing the analytes to the chemical forms that are more compatible to the chromatographic environment; creating a separation mechanism or maximizing resolution efficiency; and improving detection and structural elucidation effectiveness. Unique chemical derivatization approaches have also been applied to certain categories of analytes to meet special analytical needs.

COMPATIBILITY WITH THE CHROMATOGRAPHIC ENVIRONMENT

The majority of chemical derivatizations are performed to convert the analytes to chemical forms that are more compatible with the chromatographic environment. The bringing about of the compatibility may be mandatory or simply to improve performance characteristics. There is, however, no clear distinction between these two categories; the use of a column with a different stationary phase may render the mandatory requirement an option.

In addition to the obvious volatility concerns, carboxylic acids and amines form strong hydrogen bonds with any of the silanol groups present in the chromatographic system or components of sample residues left in the injec-

Table 1. Silylation, acylation, and alkylation derivatizing reagents and characteristics

Reagent and reaction	Characteristics[a]
Silylation	
N,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) 	Reacts faster and more completely than BSA; Combine with 1% or 10% TMCS for hindered hydroxyl and other functionalities.
Trimethylchlorosilane (TMCS) 	Commonly used as a catalyst; Reaction by-product HCl.
N,*O*-Bis(trimethylsilyl)acetamide (BSA) 	Mild reaction conditions; Forms stable products; By-product TMS–acetamide may elute with analyte.
N-Methyltrimethylsilyltrifluoroacetamide (MSTFA) 	By-product TMS–acetamide very volatile; Most suitable for volatile trace analyte.
Trimethylsilylimidizole (TMSI) 	Reacts with hydroxyl but not amine; Suitable for hindered hydroxyl group.
Trimethylsilyldiethylamine (TMS-DEA) 	Basic reagent for amino and carboxylic acids.
Hexamethyldisilazane (HMDS) 	A weak TMS donor.
N-Methyl-*N*-(t-butyldimethylsilyl)trifluoroacetamide 	Exceptionally strong yet mild reagent; Stable product in resisting hydrolysis; Combine with 1% t-butyldimethylchlorosilane catalyst for hindered alcohol and amine.
Acylation	
Anhydrides (TFAA, PFPA, HFBA, AA, TCAA)[b] 	Formation of fluoroacyl derivatives greatly increase volatility and improve detectivity in GC and MS, especially negative chemical ionization; Often used with bases, such as triethylamine.
Heptafluorobutyrylimidizole (HFBI) 	Reaction fast and mild, work best for phenol, alcohol and amine; By-product is not acidic.
N-Methyl-N-bis(trifluoroacetamide) (MBTFA) 	Reacts rapidly with primary and secondary amine, slowly with alcohol, phenol, and thiol; Mild reaction conditions with inert and volatile by-products.
Pentafluorobenzoyl chloride (PFBCl) 	Highly reactive, forming the most sensitive ECD derivatives of amine and phenol; Suitable for sterically hindered functionalities; Base often used to remove HCl produced.

(to be countinued)

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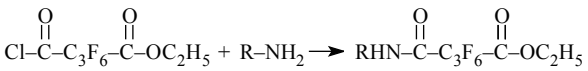
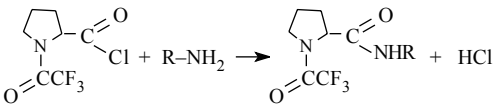
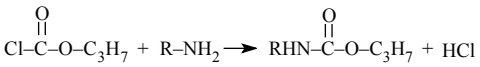
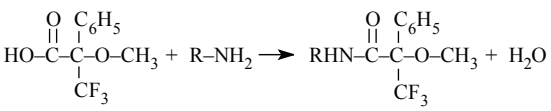
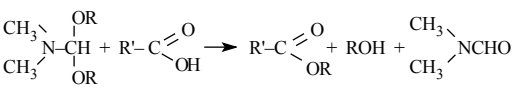
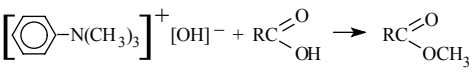
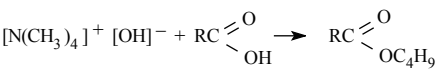
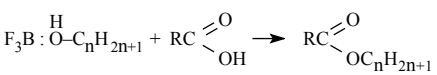
Table 1. Silylation, acylation, and alkylation derivatizing reagents and characteristics (countinued)

Reagent and reaction	Characteristics[a]
4-Carbethoxyhexafluorobutyryl chloride (4-CB) 	Form stable products with secondary amines, such as methamphetamine, allowing the removal of excess agent by adding protic solvents.
(S)-(–)-N-(Trifluoroacetyl)-prolyl chloride (l-TPC) 	Widely used for amine drugs; With a proton at the chiral center in α-position to the carbonyl group, storage and reaction conditions have to be carefully controlled to avoid racemization through keto-enol tautomerization.
Propyl chloroformate 	Fast reaction and the resulting derivatives are water soluble allowing the removal of by-products through aqueous washing.
(–)-α-Methoxy-α-trifluoromethylphenylacetic acid (MTPA) 	More effective than l-TPC in resolving ephedrines and in generating ions for designating these analytes and their isotopically labeled internal standards.
Alkylation	
DMF-Dialkylacetal (*n* = 1, 2, 3 or 4) 	Most commonly used for carboxyl groups, but also reacts with amine, phenol, and amino acid; With *n* = 1, 2, 3, or 4 to control analyte retention time.
Trimethylanilinium hydroxide (TMAH) 	Commonly used as a flash alkylation reagent.
Tetrabutylammonium hydroxide (TBH) 	Especially suitable for low molecular weight amines.
BF_3/Methanol (*n*-Butanol) (*n* = 1 or 4) 	Most commonly used to form methyl (butyl) ester with acid.

[a]Information included in this column are only for general reference purpose and not meant to be comprehensive nor critical evaluation.
[b]TFAA, PFPA, HFBA, TCAA, and AA are trifluoroacetic, pentafluoropropionic, heptafluorobutyric, trichloroacetic, and acetic anhydrides.

tor or column. These undesired interactions can result in peak loss or peak tailing caused by irreversible or reversible adsorption, respectively[2]. Thus, these hydroxyl (free or part of a carboxylic acid) or amine groups are often converted to an inactive species prior to their chromatographic analysis. The chromatograms in Figures 1A and 1B[2], obtained using a DB-5 column (5% phenyl polysiloxane phase), show the dramatic differences in their chromatographic characteristics of the six amine and alcoholic amine drugs with and without derivatization. Thus, with the DB-5 column, quantitative determinations or even qualitative identifications of these compounds cannot be achieved without prior derivatization.

The derivatization of barbiturates represents an effort to improve their chromatographic characteristics. While derivatization of barbiturates is not mandatory for their GC analysis, barbiturates in their native forms tend to cause adsorption and result in material loss, column contamination, and peak tailing (Figure 2A). Significantly improved results can be obtained[3] with N,N-dimethylation (Figure 2B) prior to their chromatographic analysis. The methylation process has also been utilized[4] to

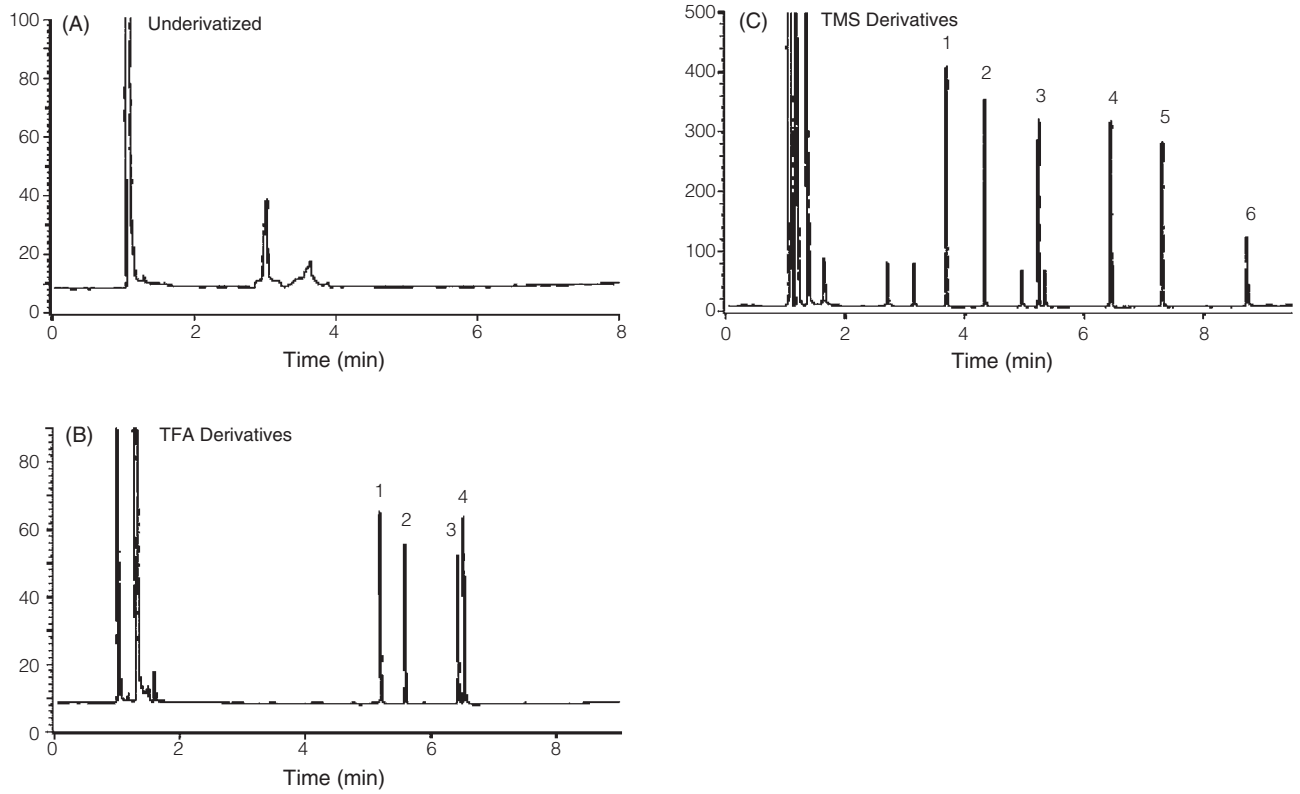


Figure 1. Gas chromatograms of a mixture of amphetamine drugs: underivatized (A); trifluoroacetyl-derivatized (B); and trimethylsilyl-derivatized (C). (Redrawn from Ref. 2.)

add additional information for confirmatory identification purposes. In this application[5], extracts obtained

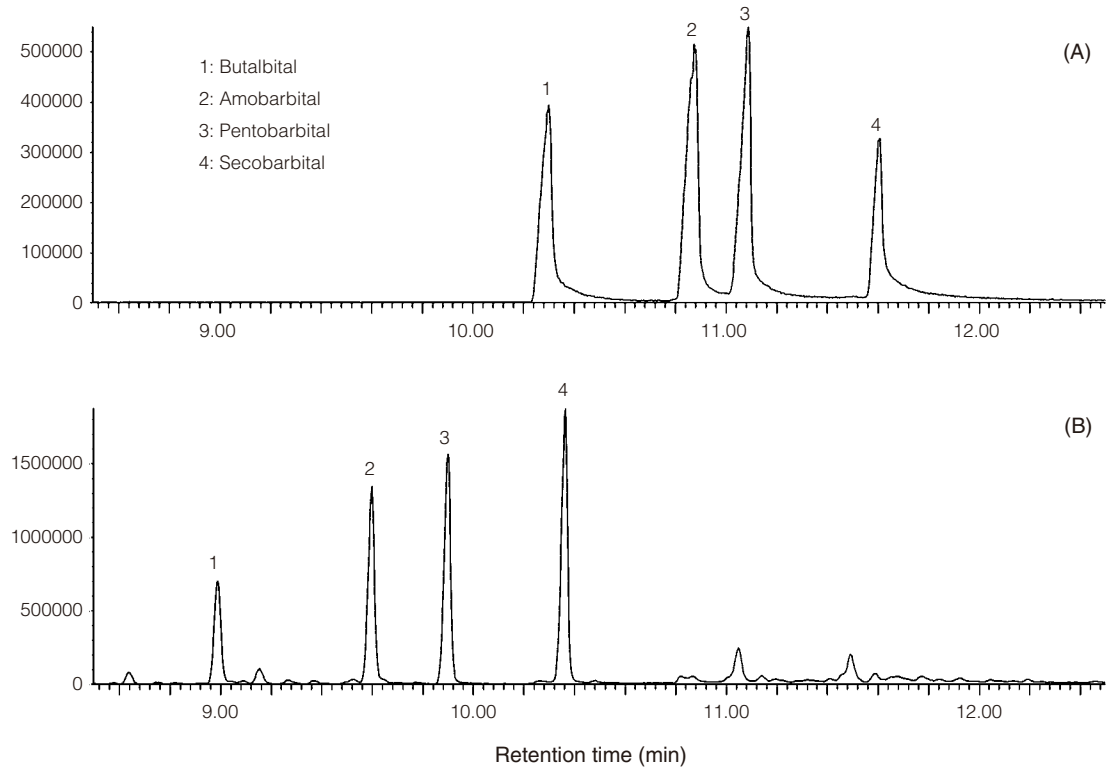


Figure 2. Total ion chromatograms of underivatized (A) and *N,N*-dimethyl-derivatized barbiturates (B).

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from urine samples screened positive by RIA were first chromatographed without derivatization; extracts that show the presence of barbiturates are then derivatized and chromatographed again. With the conformity of chromatographic parameters to the respective controls, the certainty in confirming the presence of these barbiturates is reinforced.

Another report[6] on large-scale and routine quantitative analysis of four barbiturates (butalbital, amobarbital, pentobarbital, and secobarbital) clearly demonstrated that methylation greatly improved the analysis of these compounds in the following aspects:

1. Chromatographic peak shape of these compounds was generally better, and more importantly, the interval between column maintenances, during which acceptable chromatograms were produced, were greatly lengthened;

2. Analyte stability was significantly improved as reflected by observing more consistent quantitative results from extraction/derivatization products that were delayed in their GC/MS analysis for different length following the reconstitution step; and

3. Reproducibility in the quantitation of control samples was significantly improved.

ACHIEVING REQUIRED SEPARATION OR IMPROVING SEPARATION EFFICIENCY

I. *Achieving Required Separation*

Enantiomeric separation can be successfully achieved by chiral stationary phases; however, many applications are routinely carried out using derivatization with chiral reagents. The derivatization may not necessarily add an additional step in the analytical process in cases, where derivatization with non-chiral reagents is applied to improve chromatographic characteristics even when a chiral stationary phase is used. This point is well illustrated by enantiomeric analyses of amphetamine and methamphetamine[7].

Figure 3[8] illustrated the chromatograms resulting from the combined use of a chiral derivatizing reagent and a chiral column. The four possible isomers resulting from the reaction of *d*- and *l*-amphetamine with *d*- and *l*-TPC are completely resolved using the Chirasil-Val column. This is important because commercial TPC contains a small amount of *d*-TPC. The elution order of these four isomers in increasing retention time is *N*-TFA-*d*-prolyl-*d*-amphetamine (Da-d), *N*-TFA-*l*-prolyl-*l*-amphetamine (La-l), *N*-TFA-*d*-prolyl-*l*-amphetamine (La-d), and *N*-TFA-*l*-prolyl-*d*-amphetamine (Da-l). The assignments of these four peaks in a chromatogram were based on relative peak sizes. Since the purity of the TPC reagent and the relative concentrations of *d*- and *l*-amphetamine in control samples are known, the relative intensities of Da-d, La-l, La-d, and Da-l are predictable and their corresponding peaks are assigned accordingly.

Contrarily, the four isomers resulting from the reaction of *d*- and *l*-methamphetamine with *d*- and *l*-TPC are resolved into three peaks (Figure 3B) only. Based on relative intensities and the known quantity injected, these three peaks, in order of increasing retention time, are N-TFA-*d*-prolyl-*d*-methamphetamine (Dm-d), *N*-TFA-*l*-prolyl-*l*-methamphetamine/*N*-TFA-*d*-prolyl-*l*-methamphetamine (Lm-l/Lm-d), and *N*-TFA-*l*-prolyl-*d*-methamphetamine (Dm-l). The inability of the Chirasil-Val column to resolve Lm-l and Lm-d is attributed to the replacement of the active hydrogen atom attached to the nitrogen atom by a methyl group. This replacement reduces the efficiency in forming a transient diastereomeric association complex between the substrate and the chiral phase[9].

Figure 3C is a chromatogram of an authentic amphetamine and methamphetamine mixture obtained from an achiral 25-m SP-2100 column. Since Da-l and La-d and Da-l and La-l are enantiomers to each other and not resolved by the achiral column, only two peaks are observed. By observing the relative intensity of these two peaks, it is concluded that the La-l/Da-d pair elute first. Similar assignments are applied to the metham-

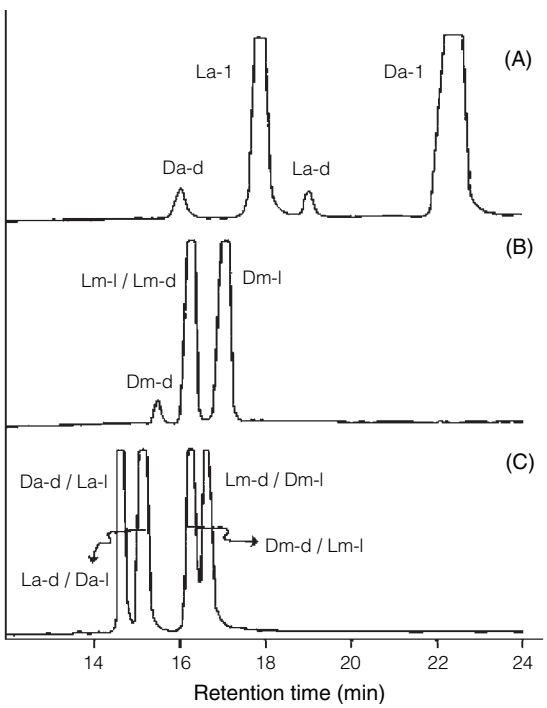

Figure 3. Total ion chromatograms of (*N*-trifluoroacetyl-*l*-prolyl-derivatized products: amphetamine (A); methamphetamine (B); amphetamine and methamphetamine mixture (C). (A) and (B) were obtained using a chiral column, while (C) was obtained using an achiral column. Compounds shown in these ion chromatograms are Da-d: *N*-TFA-*d*-prolyl-*d*-amphetamine; La-l: *N*-TFA-*l*-prolyl-*l*-amphetamine; La-d: *N*-TFA-*d*-prolyl-*l*-amphetamine; Da-l: *N*-TFA-*l*-prolyl-*d*- amphetamine; Dm-d: *N*-TFA-*d*-prolyl-*d*-methamphetamine; Lm-l: *N*-TFA-*l*-prolyl-*l*-metham- phetamine; Lm-d: *N*-TFA-*d*-prolyl-*l*-methamphetamine; Dm-l: *N*-TFA-*l*-prolyl-*d*-methamphetamine. (Reproduced with permission from Ref. 8.)

phetamine peaks.

The contribution due to the small amount of *d*-TPC can be corrected using the following equations[7]:

Aa,d = A (Aa',d – D) / (A – D);

Aa,l = A (Aa',l – D) / (A – D)

where Aa,d and Aa,l are the corrected areas for *d*- and *l*-enantiomer respectively; Aa',d and Aa',l are the apparent areas of *d*- and *l*-enantiomer obtained from the chromatograms; A = (Aa',d + Aa',l)/2; and D is the impurity (Y) of *d*-TPC in units of peak area and is given by D =Y/100 × (Aa',d + Aa',l). Thus, with known concentration (Y) of the *d*-TPC impurity in the chiral derivatization (*l*-TPC), the observed peak areas for the *d*- and *l*-enantiomers can be corrected, helpful to the determination of the exact enantiomeric compositions of *d*- and *l*-enantiomers in the test sample.

II. *Improving Separation Efficiency*

Under a high-volume production environment, targeted analytes should be eluted and well resolved within an approximately 2-6 minute retention window using a reasonably high isothermal GC column temperature. Retention time shorter than 2 minutes may be interfered by the solvent, while long retention time reduces the number of samples that can be analyzed. Isothermal operation is convenient and more reproducible; it also causes less baseline drift and minimizes chance of gas leak that may develop as a result of temperature cycling. Operating at a higher GC oven temperature helps maintain a cleaner chromatographic system.

Derivatizations are often performed to help achieve more ideal analytical condition. To bring the analytes' retention time to a more desirable range, drugs of low molecular weights may be converted to esters or amides with acids or alcohols of higher molecular weights, while drugs of higher molecular weights may be converted to esters or amides with improved volatility using fluoro-containing acids or alcohols of lower molecular weights.

The derivatization of ecgonine methyl ester and benzoylecgonine with pentafluoropropionic anhydride[6] for the simultaneous analysis of these two compounds and cocaine serves as a good example to demonstrate how chromatographic efficiency can be improved through derivatization. Although these three compounds can be chromatographed using a DB-5 column[10], the chromatographic conditions utilized and the resulting chromatogram (Figure 4A) is not as satisfactory as that obtained when a derivatization step (pentafluoropropionic anhydride) was included in the sample preparation process (Figure 4B)[6]. The latter chromatogram was obtained using a dimethyl silicone (HP-19091-6-312) fused-silica capillary column with temperature programming from 100 to 225°C at 50°C/min. Judging from the observed resolution, these three derivatized analytes can be well resolved with an isothermal operation at a reasonably high temperature.

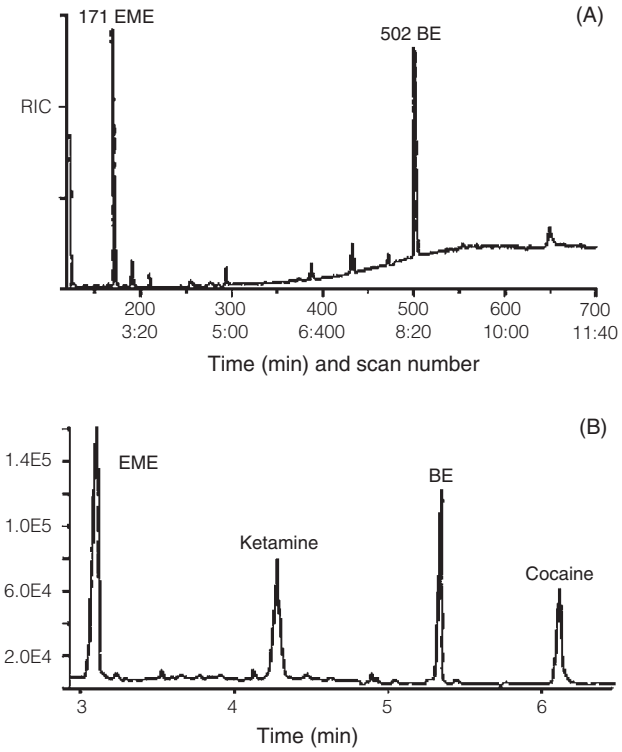

Figure 4. Chromatograms of two samples containing ecgonine methyl ester (EME), benzoylecgonine (BE), and cocaine: underivatized (A); pentafluoropropionyl-derivatized (B). (A) and (B) were redrawn from Ref. 10 and Ref. 6, respectively.)

IMPROVING DETECTION AND STRUCTURE ELUCIDATION EFFECTIVENESS

Chemical derivatizations are commonly used to enhance analyte detection, to improve quantitation, and to facilitate structural elucidation. Fluorinated anhydrides are extensively used to convert alcohols, phenols, and amines to their fluoroacyl derivatives. While enhancing analyte volatility through the introduction of fluorine atoms may be desirable in some applications, the high volatility of the resulting derivatives may prohibit the use of higher operational temperature and may not always be desirable for the analysis of low molecular-weight analytes, such as amphetamine and methamphetamine[11]. Furthermore, the negative inductive effects of the fluorine atoms in the derivatized product were found to render the products more susceptible to hydrolysis in the presence of moisture[12,13].

The introduction of these fluorine atoms, however, greatly enhances the detection effectiveness in cases where electron capture detection[14] is used. For example, an electron capture detector was used to achieve a 2-pg detection limit for heptafluorobutyryl derivative of morphine in 1977[15].

In GC/MS applications, mass spectra obtained from thoughtfully designed derivatives can show distinc-

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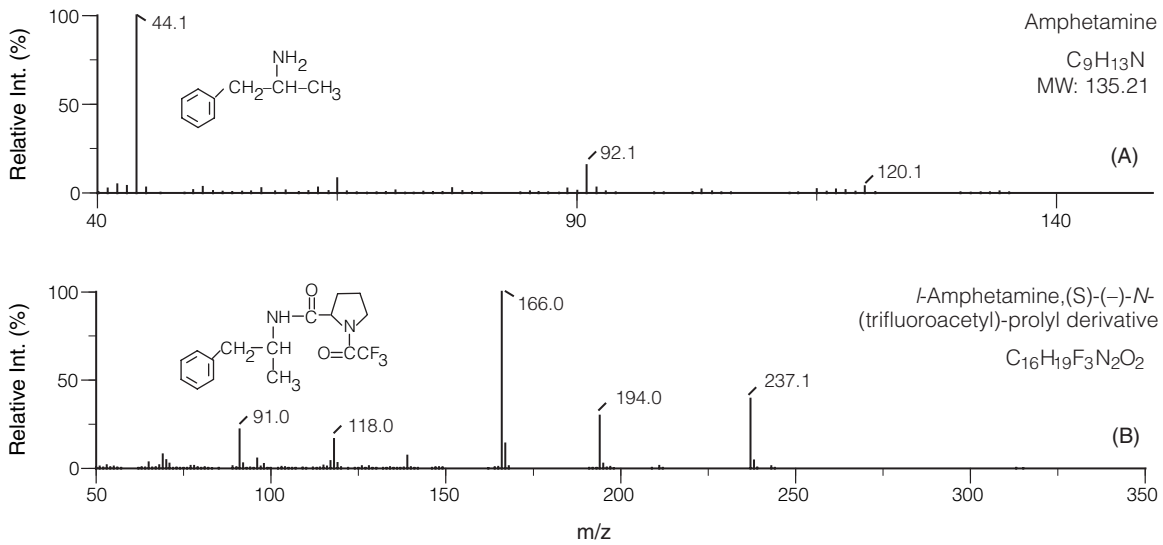

Figure 5. Electron impact mass spectra of amphetamine: underivatized (A); (*N*-trifluoroacetyl-*l*-prolyl-derivatized (B).

tive characteristics that are not available from parent compounds. The resulting advantages include the generation of ions more suitable for quantitation and helpful to structural elucidation as further discussed below.

I. *Generation of Favorable Derivative to Improve the Limit of Detection*

The formation of fluoroacyl derivatives from alcohols, phenols, and amines, an approach described early and used to improve the limit of detection in GC applications, has also been applied to negative ion chemical ionization (NICI]) in GC/MS applications[16,17]. For example, the NICI method generated a signal that is 200-fold stronger than the positive chemical ionization (PCI) counter-part when Δ9-tetrahydrocannabinol-11-oic acid is analyzed as its pentafluoropropyl/pentafluoropropionyl derivative[18]. Similarly, the NICI signals for the pentafluorobenzoyl derivative of Δ9-tetrahydrocannabinol and the pentafluorobenzoyl and tetrafluorophthaloyl derivatives of amphetamine were found to be 328-, 100-, and 678-fold, respectively, stronger than those obtained under PCI condition[16].

II. *Generation of Favorable Mass Spectra through Derivatization*

(I) *Generation of Ions More Suitable for Quantification*

Mass spectra obtained from thoughtfully designed derivatives can show distinctive characteristics not available from parent compounds. Alteration of mass spectra characteristics can result in various merits as illustrated below. For the example shown in Figure 5[18], improved detection of amphetamine can be achieved through the measurement of ions obtainable only through derivatization. The spectrum of the parent compound exhibits low intensities of ions at higher mass range. Considering the probability of contributions from interfering compounds, the low mass *m/z* 44 ion is not suitable for quantitation.

(II) *Generation of Ions Helpful to Structural Elucidation*

Chemical derivatization can be used to preserve the structural characteristics to generate mass spectra that are more amenable to interpretation. For example, to prevent ring contraction that may occur at elevated temperatures, the 3-hydroxy group in oxazepam is derivatized with trimethylsilyl[19] or alkyl[20] group in GC/MS analysis.

Mass shifts in the spectra produced by different derivatizing agents can provide extremely useful information for the identification of an unknown compound. For example, the number of trimethylsilyl (TMS) groups attached to the parent compound is deduced based on the mass shifts resulting from replacing *N*,*O*-bis-(trimethylsilyl)-acetamide (BSA) with d_9-BSA as the derivatizing agent[21]. This information facilitates the identification of desoxymorphine-A, monoacetyldesoxymorphine-A, and diacetyl-desoxymorphine-A as the impurities in an illicit heroin sample. The same approach is used to characterize O^6- and O^3-acetylmorphine[22].

Similarly, compared to the mass spectrum (Figure 6A) of the parent compound, the 28 amu mass shift observed in the mass spectrum of the derivatized pentobarbital (Figure 6B) indicates the replacement of 2 H's by 2 methyl groups[5].

As a third example, compared to parent compounds, TMS derivatives of *N*-substituted barbiturates are found to generate less olefin radical elimination ($[M–41]^+$ and $[M–55]^–$). Instead, the formation of the $[M–15]^+$ ion is favored, thus making it easier to recognize the molecular weight of the compound under examination[23].

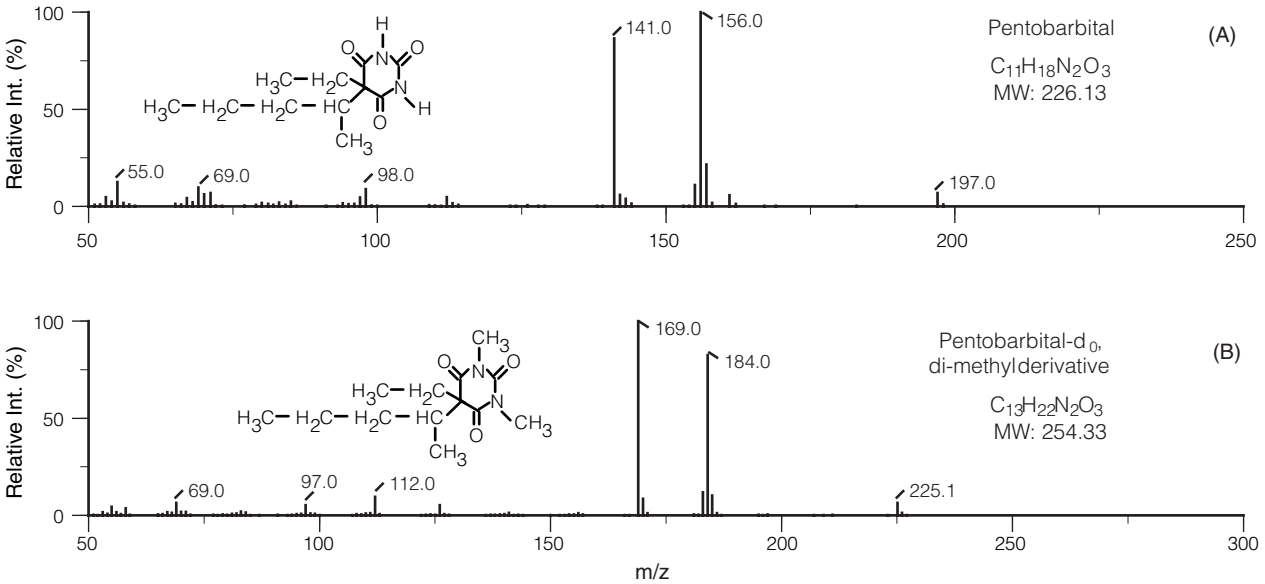

Figure 6. Electron impact mass spectra of pentobarbital: underivatized (A); methyl-derivatized (B).

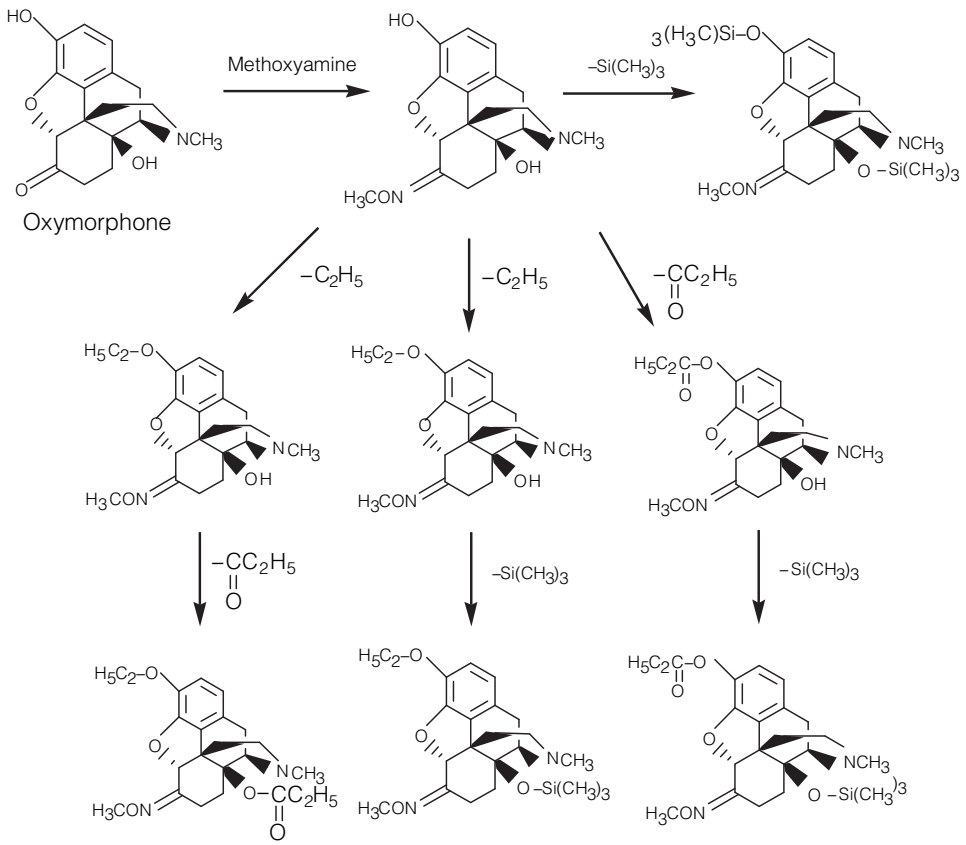

Figure 7. Scheme of a multiple derivatization approach — oxymorphone example. (Reproduced with permission from Ref. 24.)

SPECIAL APPLICATIONS

Certain analytes, such as oxymorphone, oxycodone, hydromorphone, and hydrocodone, may exist as keto- and enol-forms. The composition of these two forms may also be different dependent on the matrix acidity and other factors. The conversion of the keto-functional to an oxime, followed by subsequent conventional derivatiza-

tion approaches (Figure 7) has been well studied. This approach was found effective for simultaneous analysis of these and related compounds[24].

COMMON CHEMICAL DERIVATIZATION REACTIONS AND PRACTICAL CONSIDERATIONS

Information concerning the chemical derivatization of compounds for chromatographic and related analyses are widely available in the literature[1,25-34]. Many protocols are also provided by commercial suppliers carrying derivatizing reagents[35,36]. Since a derivatization reaction should be simple, rapid and stoichiometric, this analytical approach is applied mainly to compounds possessing labile protons on heteroatoms with functional groups such as –COOH, –OH, –NH, and NH_2 — although high-yield derivatization at carbon sites has also been reported[37].

In summary, three major categories of derivatization reactions are commonly used for drug analysis; these are silylation, acylation, and alkylation. Included in Table 1 are commonly used derivatization reagents with brief descriptions of their main characteristics.

Several practical considerations, as listed below, have also to be considered when selecting a derivatization reaction and a derivatizing reagent.

1. Safe and easy formation of the derivative with a readily available and inexpensive reagent;

2. High yield of a stable product;

3. Mild reaction conditions preventing undesirable reaction to the analyte; and

4. No undesirable by-products that may be harmful to the stationary phase.

Thus, historically important diazomethane for forming methyl ester derivatives from carboxylic acids is no longer popular. The reagent is highly toxic, the reaction is hazardous in causing explosion, and the reaction products often include artifacts of unsaturated and keto-acids.

Catalysts, such as HCl, BF_3, and BCl_3 are commonly used with alcohols to form ester derivatives of carboxylic acids. The HCl, used or formed as a by-product, when trimethylchlorosilane is used as the trimethylsilyl- (TMS-) derivatization reagent, should be removed prior to the introduction of the derivatization product to a GC or a GC/MS system. Thus, pyridine and dimethylformamide are commonly used as the solvents because they also act as acid scavengers. Similarly, triethylamine or 5% bicarbonate are used as neutralization agents when trifluoroacetic acid is formed in the trifluoroacetyl derivatization process.

Since the TMS derivatives are susceptible to hydrolysis in the presence of moisture (stability decreases in the order of TMS-ethers > TMS-esters > TMS-amines[34]), exposure of the derivatization product to the atmosphere should be limited, especially when the derivatives are not analyzed immediately.

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