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Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline

This document provides guidance on establishing uniform practices necessary to produce quality data for quantitation and identification of a drug or drug metabolite using the GC/MS method; specific quality assurance criteria for maintaining and documenting optimal instrument performance are also presented.

A guideline for global application developed through the NCCLS consensus process.

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Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline

Abstract

NCCLS document C43-A— *Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline* is intended to aid the laboratorian in developing appropriate procedures for the use of GC/MS in confirmation analyses. Its primary objective is to establish uniform practices necessary for producing quality data for quantitation and identification of a drug or drug metabolite. To support the scientific basis of the uniform practices, a brief overview of the techniques is provided. Specific quality assurance criteria for maintaining and documenting optimal instrument performance are presented.

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Contents

Abstract i

Committee Membership v

Active Membership vii

Foreword xv

The Quality System Approach xvi

1 Introduction 1

2 Scope 1

3 Standard Precautions 1

4 Principles of GC/MS 2

4.1 Gas Chromatography 2
4.2 Interface 3
4.3 Mass Spectrometry 3
4.4 Data Acquisition 7

5 Definitions 10

6 Method Validation 12

6.1 Specificity 12
6.2 Trueness 13
6.3 Precision (Repeatability and Reproducibility) 14
6.4 Linearity 14
6.5 Limit of Detection 15
6.6 Limit of Quantitation 16
6.7 Robustness 16

7 Routine Instrument and Method Performance Verification 16

7.1 Gas Chromatograph 16
7.2 Mass Spectrometer 17
7.3 Calibration 18
7.4 Quality Control/Quality Assurance 19

8 Identification Principles 20

8.1 Gas Chromatograph 20
8.2 Mass Spectral Identification 21

References 24

Additional References 26

Summary of Comments and Subcommittee Responses 27

Related NCCLS Publications 33

Foreword

The detection of a drug in the biological fluid of an individual can have serious professional, financial, and social consequences. It is generally accepted that detection of a drug by a screening procedure must be confirmed by a second method based on a different analytical or physical principle. The purpose of the confirmation test is to decrease the probability of false-positives and to provide additional information and assurance about the identity of the detected compound.

Gas chromatography/mass spectrometry (GC/MS) is widely accepted in both scientific and legal arenas as one of the most powerful analytical techniques for the separation, quantification, and identification of drug analytes, especially at low concentrations. Technological advances have allowed introduction of bench-top GC/MS instrumentation into forensic and clinical toxicology laboratories. Further advances will continue to move state-of-the-art techniques such as gas- and liquid-phase chemical ionization, tandem mass spectrometry, high-resolution mass spectrometry, and high-performance liquid chromatography/mass spectrometry (HPLC/MS) into routine laboratory operation. Appropriate application of these analytical tools requires that the methods used are fit for their purpose and the instruments are operating correctly.

The Division of Workplace Programs, Substance Abuse and Mental Health Services Administration of the United States Department of Health and Human Services oversees the best-known drug testing program. The U.S. National Laboratory Certification Program has issued guidance documents for laboratories involved in the federal workplace drug-testing program. A similar program is under consideration in the European Union. Confirmatory assays are also used in clinical toxicology, forensic toxicology, and athletic drug testing. Currently available guidelines are not appropriate for all drug confirmation testing. The present guideline was developed to provide assistance in developing confirmation tests that are fit for the analytical purpose in each of these areas.

This guideline addresses the instrumental and methodological issues in developing a chromatographic - mass spectrometric method, routine performance of the analysis, and continued quality assurance. The chain of custody, while an important part of any test result to be submitted to the judicial system, is not discussed here. Guidelines for sample collection and screening testing have been published. Refer to the most current edition of NCCLS document T/DM8—*Urine Drug Testing in the Clinical Laboratory* for recommendations on sample collection and screening testing.

Key Words

Athletic drug testing, clinical toxicology, drugs of abuse, forensic toxicology, gas chromatography, magnetic sector mass spectrometer, mass spectrometry, quadrupole mass spectrometer, tandem mass spectrometry

A Note on Terminology

NCCLS, as a global leader in standardization and harmonization, is firmly committed to achieving global harmonization wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. NCCLS recognizes that medical conventions in the global metrological community have evolved differently in the United States, Europe, and elsewhere; that these differences are reflected in NCCLS, ISO, and CEN documents; and that legally required use of terms, regional usage, and different consensus timelines are all obstacles to harmonization. In light of this, NCCLS recognizes that harmonization of terms facilitates the global application of standards and deserves immediate attention. Implementation of this policy must be an evolutionary and educational process that begins with new projects and revisions of existing documents.

Of particular note in C43-A, are several terms whereby NCCLS intends to eliminate confusion over time, through its commitment to harmonization. For instance, the term "accuracy" comprises three different concepts that ISO documents capture with three distinct terms; i.e., "accuracy," "trueness," and "bias." Also in the context of this guideline, the term "precision" is defined the way ISO defines "uncertainty." To facilitate understanding, all ISO terms are defined in the guideline's "Definitions" section under the terms "accuracy" and "precision."

All terms and definitions will be reviewed for consistency with international use, and revised appropriately during the next scheduled revision of this guideline.

The Quality System Approach

NCCLS subscribes to a quality system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents through a gap analysis. The approach is based on the model presented in the most current edition of NCCLS HS1—*A Quality System Model for Health Care.* The quality system approach applies a core set of "quality system essentials (QSEs)," basic to any organization, to all operations in any healthcare service's path of workflow. The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The quality system essentials (QSEs) are:

QSEs

Documents & Records	Information Management
Organization	Occurrence Management
Personnel	Assessment
Equipment	Process Improvement
Purchasing & Inventory	Service & Satisfaction
Process Control	Facilities & Safety

C43-A addresses the following quality system essentials (QSEs):

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessment	Process Improvement	Service & Satisfaction	Facilities & Safety
					X						

Adapted from NCCLS document HS1— *A Quality System Model for Health Care*.

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, GP26-A2 defines a clinical laboratory path of workflow which consists of three sequential processes: preanalytical, analytical, and postanalytical. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information. The arrow depicts the sequence, from left to right, that any clinical laboratory follows. In addition, the necessary steps or subprocesses are listed below them.

Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline

1 Introduction

Gas chromatography/Mass spectrometry (GC/MS) is generally accepted as the "gold standard" for identification and quantitation of drug analytes. As such, it is frequently used to confirm presumptive positive drug screening tests performed by immunoassay, thin-layer chromatography, high-performance liquid chromatography, or gas chromatography. The confidence in the ability of GC/MS to provide unequivocal analytical data is based on recognition of its reproducibility, repeatability, specificity, and trace detection capabilities. While this confidence is well founded, the measurement and identification of trace levels of compounds in complex biological matrices such as urine, hair, blood, bile, or organ tissue present a unique problem. Since GC/MS confirmation tests are applied in areas of clinical and forensic science other than drugs of abuse testing, it seems appropriate to establish broader criteria.

2 Scope

In drug analysis, GC/MS is used either to increase confidence in the identification of an unknown compound or to improve the limits of detection or quantitation through increased analytical specificity. Because of this unique combination of identification and quantitation capabilities, GC/MS methods, particularly confirmation methods, require a specific set of criteria for validation of methods and for performance verification in routine analysis.

There are two broad classes of drug analysis performed with GC/MS instrumentation. For some compounds, quantitative concentration thresholds have been established, on scientific and administrative grounds, to determine the presence of the drug or drug metabolite. When the threshold concentration, threshold ratio of amounts, or other defined parameter is exceeded, the compound is deemed to be present or to be present in nonphysiological amounts. In these cases, the performance of the method and instrument at the threshold has particular importance. The best-known example of the threshold approach was the development of specific administrative threshold concentrations and criteria for identification of five drugs of abuse for the federal drug-testing program.[1] For some other drugs or drug metabolites, however, detection at any documentable concentration is of concern. For these nonthreshold compounds, performance criteria for identification may be more important than the ability to quantify.

Although bench top GC/MS instrumentation has become more available and easier to use, a uniform practice must be established and maintained to provide acceptable evidence in an administrative appeal hearing or legal setting. Continuing improvements in theory and instrumentation will facilitate the use of new techniques, such as GC/MS/MS, in routine analysis. Thus, there is a need to define uniform practices not only for routine GC/MS methods, but also for the application of these more sophisticated approaches.

3 Standard Precautions

Because it is often impossible to know what might be infectious, all human blood specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are new guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996;Vol 17;1:53-80), (MMWR 1987;36[suppl 2S]2S-18S), and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials and for recommendations for the management

of blood-borne exposure, refer to NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections*.

4 Principles of GC/MS

GC/MS is one of a number of hybrid techniques that couple two analytical techniques to achieve a synergistic improvement in analytical performance. The appropriate operation of each technique is necessary to achieve an analytical performance objective.

4.1 Gas Chromatography

GC separates molecules by differences in their equilibrium distribution between a gaseous mobile phase and a liquid or solid stationary phase. The degree of separation between the different components in a mixture is affected by the mobile phase, its flow rate, the stationary phase used, and the temperature and/or rate of temperature change. The emergence of the analyte from the column gives rise to a Gaussian- shaped chromatographic peak. The time that has elapsed between injection and the time when the chromatographic peak apex appears, corresponding to the elution of the maximum concentration of analyte, is called the "retention time." If an unknown compound has the same retention time as a reference material under the same chromatographic conditions, the result is consistent with the presumption, but does not prove, that the two compounds are the same.

4.1.1 Sample Introduction

Because the analyte must be in the gas phase for separation, the sample introduction system must transform the liquid sample into a gas. There are a variety of sample introduction techniques presently available in GC, including isothermal split or splitless, temperature-programmed split or splitless, direct, and on-column. Each technique has advantages and disadvantages. All techniques require the sample entry port to be sealed to isolate the mobile phase stream from the outside environment. This requires either a septum or an alternate sealing system that is repeatedly penetrated by a needle. Because most injection techniques require sufficient heat to rapidly vaporize both the injection solvent and the analyte(s), components dislodged and volatilized from the septum can cause problems. Small amounts (ng) of analyte are introduced into the column. Due to the relatively large surface area of the injection port, its design, the inertness of the materials used, and the cleanliness of those portions which come in contact with the sample are critical for efficient, reproducible sample introduction.

4.1.2 Columns

Two types of GC columns exist: packed and open tubular (capillary). Packed columns are very infrequently used in conjunction with mass spectrometers and will not be discussed. Capillary columns are small diameter (0.10 to 0.53 mm inside diameter) tubes of fused silica. The stationary phase is a cross-linked annulus on the inner wall of the tube. The stationary phase is a polysiloxane or polysilarylene polymer backbone on which functional groups such as methyl, phenyl, cyanopropyl, or trifluoroacetyl provide sites for interaction with the compounds to be separated. The amount and polarity of the stationary phase, the temperature of the column, and the mobile phase flow rate are the major determining factors for separation of two compounds.

With respect to GC/MS, the major impact of the column, other than the ability to separate the compounds of interest, is the continuous degradation and elution of the phase, called "column bleed." Since all compounds entering the mass spectrometer contribute to the final signal, column bleed contributes to system background. Column bleed can be minimized by operating the column within the manufacturer's temperature range limits, by excluding oxygen and other contaminants from the mobile phase, by introducing samples that do not degrade the phase, and by selecting polysilarylene-based bonded phases.

4.2 Interface

The interface provides continuous introduction of the gaseous chromatographic effluent into the mass spectrometer ion source. Jet separator, membrane separator, or effusion separator interfaces were used to enrich the ratio of sample to carrier gas. Wide-bore (megabore), open-tubular GC columns generally require an "open-split" interface in which a portion of the effluent is discarded without enrichment of the sample relative to the carrier gas. With the widespread use of narrow-bore capillary GC columns, all of the column effluent can be directed into the ion source using a direct interface. In general, the interface is relatively trouble-free, although careful temperature control of the transfer line between the gas chromatograph and mass spectrometer is required.

4.3 Mass Spectrometry

The ability of mass spectrometry to obtain information related to the structure of the compound complements the separation capabilities of GC. Molecules entering the mass spectrometer are ionized and may undergo fragmentation. The pattern of fragments and their relative amounts are characteristic of the chemical structure of a compound but may not be unique for that compound. A mass spectrum is the two-dimensional plot of the relative abundance versus the mass-to-charge ratio of the ions. When the abundances of all ions in the mass spectrum are summed and plotted as a function of elution time, the plot is called a "total ion chromatogram" (TIC). An ion chromatogram is the two-dimensional plot of the abundance of a particular mass-to-charge ratio (m/z) versus the retention time of a GC/MS run.

4.3.1 Ion Source

As the GC effluent enters the ion source, either electron or chemical ionization achieves a continuous production of ions. The ion beam produced from the content of the effluent is directed to the mass analyzer by one or more electronic lenses. The lenses also provide the ions with a relatively homogenous momentum or velocity, which is important for separation of the ions in the mass analyzer.

The extent of ionization can be affected by the ionization (or filament) current, since this parameter is related to the number of electrons emitted from the filament. The temperature of the ion source may also affect fragmentation due to the kinetic nature of the ionization processes.

In the case of some ion trap designs, the ions are produced in the mass analyzer itself.

4.3.1.1 Electron Ionization

In electron ionization (EI), a beam of electrons directly bombards the GC effluent. When an electron from the filament has a near-collision with the analyte, an electron is abstracted from the molecule, resulting in the formation of an energetic cation radical. The cation radical is called the "molecular ion." The most common electron beam energy is 70 eV, since an electron with this amount of energy causes ionization of essentially all organic molecules. The cation radical can undergo a predictable and relatively reproducible fragmentation, which results in a cation and a radical. The cations formed from bond cleavage reactions are called "fragment ions." The most abundant ion is called the "base peak."

4.3.1.2 Chemical Ionization

In the chemical ionization (CI) mode, a reagent gas is introduced into a specially designed ion source. The reagent gas is bombarded and ionized by the electron beam. The reagent gas ions react with the GC effluent and chemically ionize the sample. The most common reagent gases are methane, ammonia, and isobutane. The most common types of CI reactions resulting in positive ions are proton transfers. The appearance of a protonated molecular ion and any fragmentation is a function of the gas phase proton affinity of the analyte and the reagent gas. Chemical ionization is a low-energy process and there is

usually little fragmentation in CI as compared to EI. This can decrease the amount of identification information present in the spectrum.

Negative ions can be produced either by electron capture of thermalized electrons or reaction with proton-abstracting reagents such as O^-. The former has become a method of choice for molecules containing electronegative atoms, such as the halogens contained in the benzodiazepine drug class. Negative ion chemical ionization (NICI) should be clearly indicated, since by convention CI is used for positive ions.

The ion trap presents a unique situation for CI. Because ions are stored in the ion trap, the reagent gas ions have a much longer time to react with the analyte. Thus, a much lower pressure (concentration) of reagent gas is necessary to produce an ion. This has made possible the use of more chemicals, such as acetonitrile and tetrahydrofuran, as reagent gases. Unfortunately, if ionization takes place in the ion trap, there is no opportunity to generate thermalized electrons, and thus electron-capture NICI is not possible.

4.3.2 Mass Analyzer

The mass analyzer separates the ion beam generated in the source into its component parts on the basis of their mass-to-charge ratio (m/z).

4.3.2.1 Magnetic and Electrostatic Mass Analyzers

In a magnetic analyzer, ions produced continuously in the source are accelerated toward a magnetic field by a kilovolt potential such that all of the ions have the same kinetic energy. The magnetic field, which describes a sector of a circle, separates the ion beam into its components according to their momentum. Ion beams of specific m/z are separated from each other spatially, with each beam having a unique radius of trajectory which depends on the accelerating voltage and the magnetic field radius and strength. The ion beam with a selected m/z passes through an adjustable slit and impinges on an electron multiplier where it is detected. Mass resolution can be adjusted by changing the width of the slit. Ions of different m/z can be focused on the detector by changing the field strength of the electromagnet.

An electrostatic analyzer can focus charged particles according to their kinetic energy-to-charge ratio. Since kinetic energy is related to the mass of the particle, the trajectory of an ion of particular m/z is a function of the accelerating voltage, and the radius and strength of the electrostatic field. Thus ions of specific m/z can be focused on the detector by varying the electrostatic field strength. The combination of a magnetic and an electrostatic analyzer results in a "double-focusing" instrument, which has improved mass resolution since the two analyzers use complementary ion separation principles. Detection of an ion of particular m/z can be achieved by varying either the electric or the magnetic field strength. Ion transmission and focusing can be improved by additional sectors, which can result in "triple-focusing" instruments. All commercial sector mass analyzers are double- or triple-focusing instruments.

Sector mass analyzers are important for several reasons. First, they can achieve mass resolution sufficient to determine the atomic composition of an ion. Second, because of the high accelerating voltages, almost all of the ions formed in the source are transmitted to the detector. Thus, tuning in a sector instrument involves adjusting the magnetic and electrostatic fields to transmit the correct mass, but relative ion intensities are not adjustable. Mass spectra obtained from a sector instrument are thus considered to be the "gold standard." Finally, because of the efficiency of ion transmission, a sector instrument has the highest sensitivity and lowest limits of detection of the commonly used mass analyzers.

4.3.2.2 Quadrupole Mass Spectrometer

The most popular mass analyzer is the quadrupole mass spectrometer (QMS). Ions produced continuously in the ion source are accelerated into the aperture between two pairs of parallel rods. Direct-current (DC)

and radio frequency (RF) voltages on opposite pairs of rods deflect the ion in a plane perpendicular to its movement down the long axis of the rods. The DC voltage, RF voltage and frequency, and the geometry of the rods determine the m/z ratio of the ion whose stable trajectory allows passage through the filter. Ions of other m/z either collide with the rods or are pumped away by the vacuum system. A selected m/z ion can be transmitted through the rods by selecting the appropriate DC and RF voltages. By simultaneously increasing the DC and RF voltages in a fixed ratio, ions of increasing m/z are sequentially transmitted to the detector.

The voltages applied to accelerate the ions from the source must be low (e.g., a few volts) so that the ions spend sufficient time in the quadrupole field to obtain good mass resolution. This can result in decreased transmission efficiency for high mass ions. In addition, the absolute DC and RF voltages applied and their ratio during the scan influence the transmission efficiency and apparent width of the mass peak detected. Thus, in tuning a QMS in the scan mode, the trueness of the mass axis, the relative intensities of the ions, and the width of the mass peak (and thus mass resolution) can be adjusted. This has significant implications for mass spectral library searching and matching.

Transmission of a specific ion is a different process. Because the transmission of ions with other m/z is not of concern, DC and RF voltages are chosen which generate the largest chromatographic peak profile, usually by selecting a specific mass (e.g., 432.3). In addition, the DC/RF ratio can be selected to transmit more ions at the cost of decreased mass resolution. This will increase signal and may increase signal-to-noise ratio if no ions of similar m/z are in the background or matrix. It may be appropriate in this operating mode to select targeted tuning conditions that will not result in a spectral scan with accepted relative ion intensities for a reference compound like perfluorotributylamine (PFTBA).

4.3.2.3 Ion Trap

The ion trap also separates ions through their interaction with a quadrupole field generated by RF and DC voltages. The ion trap can operate in several modes: selected mass detection, selected mass storage, and selected mass ejection. In the selected mass ejection mode used on most commercially available ion traps, all ions formed during ionization are stored within a space surrounded by the ring and end-cap electrodes by application of an RF voltage to the ring electrode. A linear increase in RF voltage causes instability in the trajectory of ions of increasing m/z which results in their ejection from the ion trap. The ions are detected with an electron multiplier located outside of the ion trap. A variety of other electronic wave functions can be applied to the end-cap electrodes of the ion trap in order to improve performance or facilitate ion reactions such as mass spectrometry/mass spectrometry.

4.3.2.4 Time-of-Flight Mass Spectrometers

Time-of-flight (TOF) mass spectrometers separate ions based on the time required for the ions to travel a defined distance after acceleration in an electrical field. Higher-mass ions have lower velocities than lower-mass ions. One advantage of TOF MS is the extremely rapid scan speed that can be used in conjunction with “fast GC.” The TOF analyzer is also capable of higher-mass resolution than the QMS, depending on the speed and handling of data acquisition. Due to the nature of the data acquisition, TOF MS instruments always operate in the full-scan mode.

4.3.3 Mass Spectrometry/Mass Spectrometry

Mass spectrometry/mass spectrometry (MS/MS) has been a relatively recent addition to analytical methodology. In this approach, a molecule is ionized and the molecular ion or a fragment ion is separated from other ions in a mass analyzer as described above. This precursor ion (also known as a “parent ion”) is then focused into a collision cell where it undergoes an energetic collision with a target (or collision) gas. The collision energy is partially transformed into potential energy, which results in fragmentation to form product ions (also known as “daughter ions”). This process is called “collision-induced dissociation”

(CID). The product ions are then analyzed and either a spectrum of product ion mass abundances or a selected reaction product can be monitored. The main experimental parameters that affect the CID process are the collision energy, determined by voltages within the instrument, and the collision gas thickness (or pressure).

There are two fundamentally different approaches to MS/MS employing either kilovolt collision energies (magnetic/electrostatic mass analyzers) or volt collision energies (quadrupole and ion trap mass analyzers). Because of the energy differences, the spectra obtained are frequently different. High- energy CID applications often use H_2 as the collision gas. The collision cell is usually an open design, since the velocity of the precursor ion and the low mass of the collision gas result in relatively little scattering of the product ions. In contrast, most low-energy CID applications use argon (He in ion traps) as the collision gas, and must use a collision cell that refocuses the product ion beam. In the QMS, the collision cell is usually a set of RF-only quadrupole rods into which the collision gas flows. This approach is sometimes called "MS/MS in space." In the ion trap, the CID process occurs between the stored precursor ion and the helium bath gas. The collision energy is provided by application of a voltage waveform to the end cap electrodes. Product ions are stored with the same RF field, and ejected using a mass instability scan. Since the product ions are not separated spatially from the precursor ions, this technique is sometimes referred to as "MS/MS in time." In the ion trap, the collision gas thickness cannot be varied, but the time for reaction between the precursor and the collision gas can be controlled to vary the CID product ions.

Although MS/MS spectra can be obtained, there are no spectral libraries and the rational explanation of the fragmentation processes is not as well developed as for electron ionization MS. Thus, the main analytical application of GC/MS/MS to toxicology is selected reaction monitoring (SRM), where several product ions are selectively monitored as a function of time. Comparison of several product ion abundances between a reference standard and an unknown under the same GC/MS/MS conditions should allow the development of identification criteria.

It should be noted that introduction of another collision process and mass analysis could give rise to an MS/MS/MS spectrum or third-generation product ion for quantitation. It is also noteworthy that sector instruments are capable of monitoring metastable ions. In this approach, unstable ions produced in the ion source can fragment in the field free region between the ion source and the magnetic or electrostatic mass analyzer. Since the resulting metastable ion has the momentum of its precursor but the mass of the fragment, it gives a unique "apparent" mass. Monitoring of metastable ions can provide selectivity similar to that obtained from MS/MS. Because quadrupole and ion trap analyzers do not use the same mass separation principle, metastable ions are not observed.

4.3.4 Vacuum System

The dependence of the mass separation on the specific trajectory of an ion through the mass analyzer means that the ion cannot collide with any other molecules along its path. The observed pressure in a container is the result of molecules colliding with the walls of the container or each other. Therefore mass separation devices must be operated at a sufficiently low pressure that the path the ion takes from the source to the detector is shorter than the distance between collisions with other molecules. The relationship between pressure and the mean-free path is well established. The longer the path through the analyzer, the lower the operating vacuum requirements.

There are two exceptions to the requirement for high vacuum in a mass analyzer. It has been clearly demonstrated that the presence of a low-molecular-weight gas, such as helium, in the ion trap resulted in improved mass resolution. This is the result of the helium bath gas decreasing the energy dispersion of the ions in the trap, and moving them toward the center of the ion trap where they were more efficiently ejected. A similar finding of improved transmission efficiency and mass resolution was recently demonstrated for higher pressures in a collision cell for low-energy MS/MS.

The presence of large amounts of air from a leak can influence ionization efficiency, mass resolution, and system sensitivity. It can also cause electrical arcing and system failure, particularly in systems in which high voltages are used. In smaller amounts, it can increase the need for routine maintenance due to detrimental reactions with the filament, column, or other components of the system.

4.4 Data Acquisition

The use of a mass analyzer provides the ability to acquire and analyze data in a variety of ways (see Figure 1). In the scan mode, the components of the total ion beam having a specific m/z are focused sequentially on the detector. The width of the mass peak focused on the detector depends on the mass analyzer used and the computer control of the electronic signal processing. For quadrupole analyzers, from six to ten collections are made across a one-mass unit window. For double-focusing instruments, the ion optics and the width of the collection slit determine the mass resolution. Unless scan times must be minimized, the spectrum should be collected from above the ions observed from air (m/z 35 to 50), to well above the expected molecular weight of the sample. In the case of CI, the scan should begin above the m/z of the highest mass reagent gas ions. The resulting plot of relative ion abundance as a function of the mass-to-charge ratio is the mass spectrum. The ion with the highest abundance in a mass spectrum is termed the "base peak" and is normalized to 100%. It is common practice to report other ion fragment abundances as percentages of the base peak height. The molecular ion ($M^{+\bullet}$), that results from detection of the radical cation formed during EI, corresponds to the molecular weight of the compound. Observation of the molecular ion is an important contributor in the identification of a compound. In cases where the molecular ion is not observed, CI can be used to determine the molecular mass.

The application of the scan mode to drug analysis is frequently limited by the fact that the target compounds are present at a concentration too low to provide a reliable mass spectrum. For magnetic and electrostatic analyzers and the quadrupole mass spectrometer (QMS), selecting a specific m/z of interest and monitoring it for defined periods of time can enhance the signal-to-noise ratio. This approach is called "selected ion monitoring" (SIM) for single-mass analyzers and "selected reaction monitoring" (SRM) for tandem-mass analyzers.

The following summarizes current thinking regarding the use of the spectral scan versus SIM mode of operation.[2]

- Qualitative analysis: Whether it is better to scan the spectral mass spectrometer repetitively over the full mass range, or to monitor only selected ions, continues to be debated. Both modes of operation have their strengths and limitations, so it is unwise to adhere rigidly to only one mode of operation. A good quality full-scan mass spectrum generally provides the best qualitative identification; however, the SIM mode generally is more sensitive and less affected by potential interferences from coeluting compounds. The specificity (certainty of identification) of a SIM assay depends on many factors including: the number of ions monitored; the uniqueness of the monitored ions; the selectivity of the extraction procedure; the type of derivative; the efficiency of the chromatographic separation; and the selectivity of the method of ionization. A well-designed SIM assay can provide a very reliable method of identification. However, it may be difficult to evaluate the reliability of a SIM assay without personal experience with the method or access to data from the analysis of a substantial number of specimens. With either mode of mass analysis, the analyte's retention time (or better, its retention time relative to a reference standard) should agree well with the analyte's expected (relative) retention time.

- Quantitative analysis: The accuracy of quantitative measurements performed by GC/MS is highly dependent on the intensity of the analyte's ion current relative to the background ion current ('noise') intensity. Acceptable quantitative measurements can be obtained from reconstructed ion chromatograms, or total ion current chromatograms obtained under full-scan data acquisition, if a

relatively high concentration of analyte is present in the specimen. However, when analyte concentrations are in the low-nanogram/milliliter range, it is generally necessary to use selected ion monitoring to obtain sufficient ion current intensity for accurate quantitation.

For the purposes and definitions of this guideline, the quantitative analysis comments relate to the analysis of threshold compounds and some nonthreshold compounds, while the qualitative analysis comments relate exclusively to the analysis of nonthreshold compounds.

The ability to confirm the identity of compounds based on the relative intensity of SIM ions was originally investigated by Sphon.[3] Using the spectrum of diethylstilbesterol as a model, a unique identification in a library of 30,000 compounds was obtained by comparing three ions from the spectrum at unit mass resolution with ±30% agreement between relative intensities of the base peak and ±35% agreement between relative intensities of the other two ions. Sphon concluded that three ions are regarded as a minimum, depending on the specificity of the ions monitored. It is important to note that the compound was not derivatized and that chromatographic resolving power of capillary GC was not available. More recent evaluation of these criteria against a larger database has confirmed the validity of the process.[4]

The fundamental basis of identification of a compound is the information content of the analytical data. Using this approach, other investigators have attempted to determine "selectivity indices" based on the inherent specificity of each step in the analytical procedure.[5] The information content of GC/MS/MS has been investigated by Fetterolf and Yost.[6]

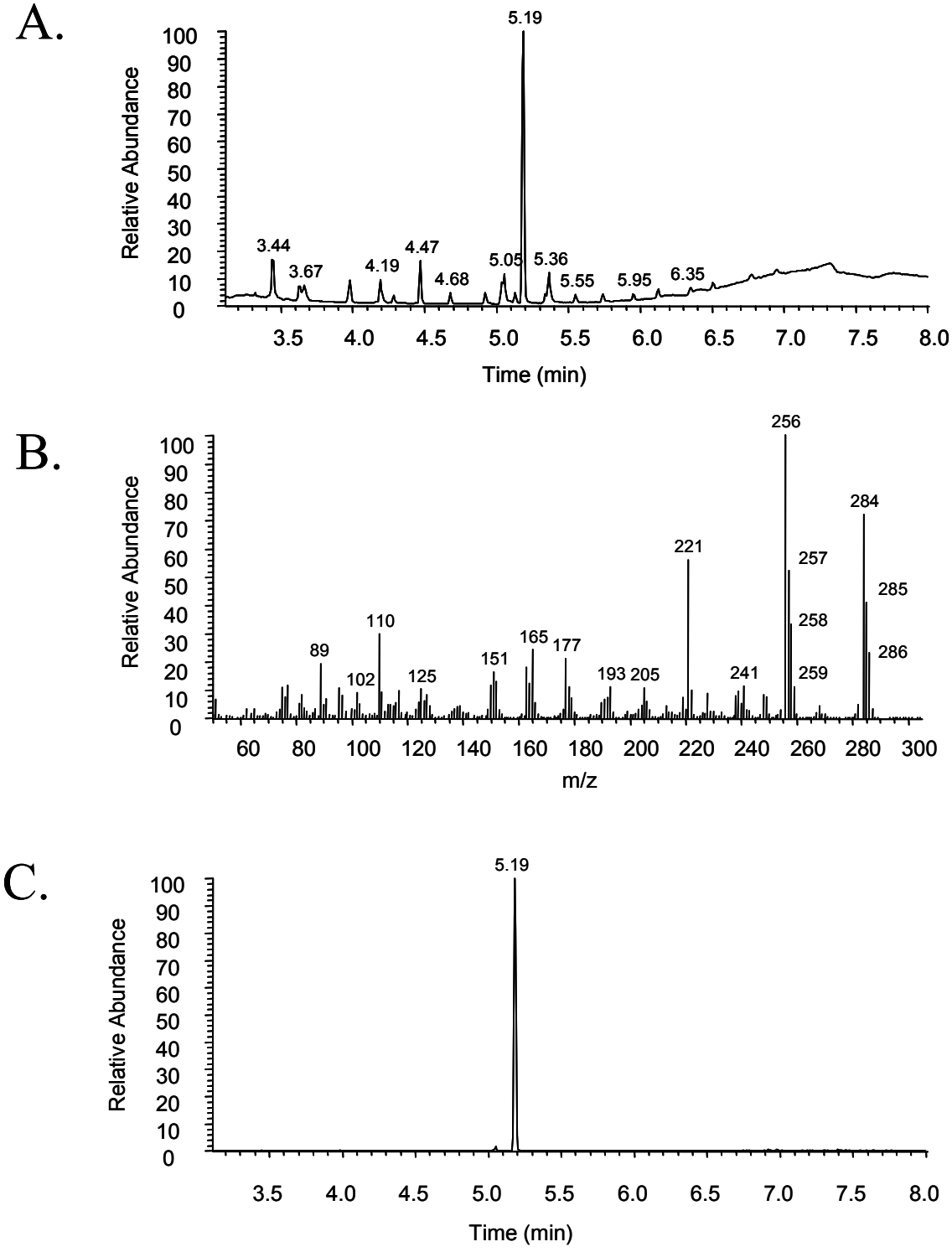

Figure 1. A) Representative GC/MS trace shown as a total ion chromatogram. B) Full scan mass spectra of diazepam, the peak that eluted at 5.19 minutes. C) Selected ion chromatogram (284 m/z) of the same data shown above.

5 Definitions[a]

Accuracy//Measurement accuracy//Accuracy of measurement - 1) Closeness of the agreement between the result of a measurement and the accepted reference value of the measurand [analyte] **NOTE:** In the context of this guideline, accuracy comprises the following three concepts, described by the following three distinct ISO 3534-1 terms: **Accuracy** - Closeness of the agreement between a test result and the accepted reference value; **Trueness** - Closeness of agreement between the average value obtained from a large series of test results and an accepted reference value; and **Bias** - The difference between the expectation of the test results and an accepted reference value; **NOTE:** In general, the deviation/difference is based on replicate measurement using an accepted (definitive, reference, or designated comparison) method and the method being tested, and expressed in the units of the measurement or as a percentage.

Bias - See Accuracy.

Best measurement capability - The smallest uncertainty of measurement a laboratory can achieve for a stated calibration under specified laboratory conditions. [7-10]

Chemical ionization, CI - The formation of new ionized species when gaseous molecules interact with ions; **NOTE**: The process may involve transfer of an electron, a proton, or other charged species between the reactants. When positive ion results from chemical ionization (CI), the term may be used without qualification; when a negative ion results, the term "negative ion chemical ionization" can be substituted. Specifics relating to ionization should be given, e.g., if negative ions are formed from sample molecules via resonance capture of thermal electrons generated in a CI source, this should be specified.[11]

Collision-induced dissociation, CID - An ion/neutral process wherein the (fast) projectile ion is dissociated as a result of interaction with a target neutral species; **NOTE**: This is brought about by conversion during the collision of part of the translational energy of the ion to internal energy in the ion.[11]

Drug - Any substance which when absorbed into a living organism may modify one or more of its functions.[12]

Drug of abuse - Drug used for a nontherapeutic purpose.[12]

Electron ionization, EI - Ionization of any species by electrons; **NOTE**: Electrons and photons do not "impact" molecules or atoms. They interact with them in ways that result in various electronic excitations including ionization. For that reason it is recommended that the terms "electron impact" and "photon impact" be avoided.[11]

Electrostatic analyzer - A velocity-focusing device for producing an electrostatic field perpendicular to the direction of ion travel (usually used in combination with a magnetic analyzer for mass analysis); **NOTE**: The effect is to bring to a common focus all ions of a given kinetic energy.[11]

Extracted ion chromatogram, EIC - Describes the processing of data from a mass spectrometer in which the ion current at one (or several) m/z values acquired in the spectral scan mode are selected and displayed as a function of time.[11]

Ion current - The intensity of an ion beam produced in the source, passed through the mass analyzer, and measured by the detector.[11]

[a] Some of these definitions are found in NCCLS document NRSCL8—*Terminology and Definitions for Use in NCCLS Documents.* For complete definitions and detailed source information, please refer to the most current edition of that document.

Ion ratio - The ratio of signal intensities at two m/z values, usually expressed as a percentage; **NOTE**: The ratio may be determined from the ratio of chromatographic peak areas or peak heights, or may be calculated from a single mass spectrum.[11]

Limit of detection, LOD - **1)** The smallest amount or concentration of analyte that can be distinguished from background at a stated confidence level; **NOTE**: For GC/MS or GC/MS/MS confirmation analysis, the compound must also satisfy identification criteria in order to be deemed detected; **2)** the lowest amount of analyte in a sample which can be detected but not quantified as an exact value.[7-10]

Limit of quantitation, LOQ - The lowest amount of analyte in a sample that can be quantitatively determined with {stated} acceptable precision and {stated, acceptable} bias under stated experimental conditions.[7-10]

Magnetic analyzer - A direction-focusing device that produces a magnetic field perpendicular to the direction of ion travel; **NOTE**: The effect is to bring to a common focus all ions of a given momentum with the same mass-to-charge ratio.[11]

Mass spectrum - A spectrum obtained when ions (usually in a beam) are separated according to the mass-to-charge ratios of the ionic species present; **NOTE**: This plot is a graphical representation of m/z versus the measured abundance information.[11]

m/z - An abbreviation used to denote the dimensionless quantity formed by dividing the mass of an ion by the number of charges carried by the ion; **NOTE**: It has long been called the "mass-to-charge ratio" although m is not the ionic mass nor is z a multiple of the electronic charge, e^-.[11]

Nonthreshold substance - A compound for which detection and identification of any amount of compound is considered to be a "positive" or "present."

Peak resolution, R_s - The separation of two peaks ($t_{R2}>t_{R1}$) in terms of their average peak width at base (w_b): $R_s=2(t_{R2}-t_{R1})/(w_{b1}+w_{b2})$; **NOTE**: In the case of two adjacent peaks it may be assumed that $w_{b1}=w_{b2}$, and thus, the width of the second peak may be substituted for the average value: $R_s=(t_{R1}-t_{R2})/w_{b2}$.[13]

Plate number - A number indicative of column performance, calculated from the following equations which depend on the selection of the peak width expression:

$$N = 5.545\ (V_R/w_h)^2 = 5.545\ (t_R/w_h)^2$$

In these expressions the units for the quantities inside the brackets must be consistent so that their ratio is dimensionless: i.e., if the numerator is a volume, then peak width must also be expressed in terms of volume.[13]

Precision - In the context of this guideline, precision is defined the way ISO 3534-1 defines **Uncertainty** (defined below).

Precursor ion - An electrically charged molecular moiety which may dissociate to form fragments, of which one or more may be electrically charged, and one or more are neutral species; **NOTE**: A precursor ion may be a molecular ion or an electrically charged fragment of a molecular ion.[11]

Product ion - An electrically charged product of a reaction of a particular precursor or parent ion; **NOTE**: In general, such ions have a direct relationship to a precursor ion and indeed may relate to a unique state of the precursor ion.[11]

Radical ion - An ion containing an unpaired electron that is thus both an ion and a free radical; **NOTE**: The presence of the odd electron is denoted by placing a dot alongside the symbol for the charge.[11]

Selected ion monitoring, SIM - Describes the operation of a mass spectrometer in which the ion currents at one (or several) selected m/z values are recorded rather than the entire mass spectrum; **NOTE**: The use of terms "multiple ion detection (MID)," "multiple ion (peak) monitoring (MPM)," and "mass fragmentography" are not recommended.[11]

Selected reaction monitoring, SRM - Describes the operation of a tandem mass spectrometer in which the product ion currents at one (or several) selected m/z values are recorded rather than the entire mass spectrum.[11]

Sensitivity (analytical) - Change in the response of a measuring system or instrument divided by the corresponding change in the stimulus (e.g., analyte concentration).[7,8,9,10]

Analytical specificity - ***In Quantitative Testing***, the ability of an analytical method to determine only the component it purports to measure or the extent to which the assay responds only to all subsets of a specified analyte and not to other substances present in the sample.

Tandem mass spectrometry, MS/MS - A technique in which an ion at a particular m/z value is isolated in one mass analysis procedure, caused to undergo fragmentation, and the products of the fragmentation process analyzed in a second mass analysis procedure; **NOTE**: The use of the abbreviation "TMS" is discouraged.[11]

Threshold substance - A compound for which a concentration has been specified, on either an administrative or scientific basis, above which the compound is deemed to be "positive" or "present" and below which the compound is deemed to be "negative" or "not detected."

Total ion chromatogram, TIC - The sum of all the separate ion currents carried by the different ions contributing to the mass spectrum plotted as a function of time.[11]

Trueness - See **Accuracy**.

Uncertainty - An estimate attached to a test result which characterizes the range of values within which the true value is asserted to lie.

6 Method Validation

All methods must be validated in a manner appropriate for the final use of the data generated by the method. A GC/MS confirmation method need not be specific for a single compound. When multiple drugs and metabolites are confirmed in the same method, validation data must be available for each compound. For threshold compounds and nonthreshold compounds for which quantitative data is determined, validation of the procedure should include all of the following parameters. Nonthreshold substances for which only detection and/or identification are reported should have specificity, limit of detection, and robustness determined, when possible.

Validation studies should be repeated if any change is made to the procedure that could affect the results.

6.1 Specificity

An investigation of specificity should be conducted during the validation of the assay. The confirmation test should be able to discriminate between compounds of closely related structures that are likely to be present. The specificity of an assay can be established by obtaining negative results in a suitable number

of known negative samples, combined with positive results in either known positive samples or negative samples spiked with a traceable reference material. The specificity of the assay may also be determined by comparison of results to those obtained by a well-characterized reference method.

In addition, the assay should be performed with compounds structurally similar to or closely related to the analyte to confirm that a positive response is not obtained. The choice of potentially interfering materials should be based on reasonable scientific judgment with a consideration of the interferences that could occur. Elimination of a compound from consideration as an interference may also be based on scientific judgment and the structure of the potential interference.

It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte. For identification of nonthreshold substances, where the screening procedure might have similar characteristics, this may be an important consideration. In this case, a combination of two or more analytical procedures (different extraction, different chromatographic conditions, different derivative, etc.) is recommended to achieve the necessary level of specificity.

6.1.1 Specific Recommendations for Nonthreshold Compounds

In circumstances where a traceable reference standard is not available (e.g., a metabolite of a drug), the use of either a biological fluid obtained after a documented ingestion of the drug of interest or a metabolite isolated from a biological sample is acceptable as a secondary reference material. The compound should be characterized to verify its identity. Comparison of GC/MS data obtained from analysis of the biological fluid or isolate in the laboratory to mass spectra published in a reputable scientific journal is acceptable verification.

The secondary reference material should be analyzed in the same analytical run as the unknown.

6.2 Trueness

Trueness is the ability of the analytical procedure to measure the true concentration or amount of analyte. The measure of trueness is called bias. There are several methods of determining bias: repeated measurement of an analyte of known purity (e.g., a reference compound); or comparison of the measured results of the proposed analytical procedure to those of a second, well-characterized procedure whose trueness is stated or defined. Comparison of measured results of the proposed analytical procedure to those obtained as a mean of a proficiency testing survey can be used to measure bias but should be used with caution because proficiency testing results are subject to non-specific and unpredictable matrix interference. Data for determining the bias of a procedure may be obtained concurrently with precision, linearity, and specificity data.

Bias should be assessed over the entire range of expected concentrations. A mean of at least three determinations at each concentration should be used to assess bias. The bias of the method should be determined for at least three concentrations. It is recommended that one of the concentrations be near the threshold concentration, where appropriate.

These measures of trueness should be evaluated at least every twelve months or when major maintenance is performed.

6.2.1 Carryover

Contamination of a sample with residual drug from another sample or standard seriously degrades the accuracy of the analysis. Procedures should be developed and instrumental conditions should be selected to minimize or eliminate carryover. Carryover can originate from the injection syringe, wash reservoir solvents, injection port, or column. The extent of carryover should be determined under specified

conditions. In the event of potential carryover during an analytical run, the procedure should contain specific instructions for eliminating carryover and obtaining an accurate result. An example of such a procedure would be inclusion of a blank injection prior to injection of each sample of interest.

6.3 Precision (Repeatability and Reproducibility)

Repeatability is the ability of the method to provide closely similar results for the same measurand under the same conditions of measurement in a short time frame. Repeatability is sometimes called within-run imprecision. Reproducibility is a measure of imprecision when the conditions of measurement vary across time, technicians, or laboratory equipment. There are within-lab and between–lab measures of reproducibility. Within-lab reproducibility can also be called between-run reproducibility. Reproducibility between laboratories is frequently assessed by means of an interlaboratory trial or by measurements of a control material over time.

6.3.1 Specific Recommendations for Threshold Compounds

Repeatability (within-run precision) should be assessed at a minimum of three concentrations covering the specified range for the procedure using a minimum of three replicates at each concentration.

Reproducibility should be estimated when multiple instruments and analysts are used in a procedure.

Both within-run and between-run precision can be determined simultaneously using appropriately designed experiments with multiple determinations on each of several days. Appropriate analysis of variance experiments can identify the relative contribution of several sources of variability in a method.

These measures of precision should be evaluated at least every twelve months or when major maintenance is performed.

6.4 Linearity

Linearity should be established by visual evaluation of a plot of peak height or area (or in the case of an internal standard method, peak height ratio or area ratio) as a function of analyte concentration. For establishing linearity, a minimum of five concentrations is required. Linearity should be established across a range of concentrations that reflects those expected in the biological matrix.

If there is a linear relationship, test results should be evaluated by calculation of a regression line by the method of least squares. Data from the regression line may also be useful to provide mathematical estimates of the degree of linearity. The residuals from the regression line as a function of concentration should be evaluated for a random distribution to support a linear relationship. The correlation coefficient can have serious limitations, particularly if the concentration range investigated is greater than one order of magnitude.[14] Despite this limitation, the correlation coefficient is a useful indicator of linearity if it is greater than 0.998.[15] An alternative to this is to plot the response factor (peak area or height divided by concentration) as a function of concentration, which should be a constant. A more robust and technically difficult method has been described.[16]

The linear range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, bias, and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. The linear range should be re-evaluated every twelve months or when major maintenance has been performed.

The standard concentrations for the calibration curve should be selected to minimize the uncertainty of the linear regression line near the threshold concentration. The best precision (lowest uncertainty) about

the regression line occurs at the mean of the standard concentrations. Where practical, the standard concentrations for the analysis should be selected so that their mean lies near the threshold concentration.

For more information on evaluating an instrument or quantitative analytical method on the basis of the manufacturer's linearity claim, see the most current version of NCCLS document EP6—*Evaluation of the Linearity of Quantitative Analytical Methods.*

6.5 Limit of Detection

A variety of methods have been proposed to determine the limit of detection (LOD) or best measurement capability.[17-24] Most of these methods rely on signal strength from a single channel of analytical data and the ability to distinguish a difference between the signal and the background at some level of confidence.[17] The LOD depends strongly on the matrix background "noise" as well as instrumental noise. In determining the LOD, the method should consider the probability of false-positive and false-negative results as well as true positive and negative results.

GC/MS provides multichannel detection capability by virtue of the simultaneous detection of multiple m/z ratio ions as a function of retention time. As a result, GC/MS data contain both identification and quantification information. For the purposes of this guideline on confirmation, detection requires both a signal discernable from the background and satisfaction of identification criteria used for the method.

Historically, two methods have been used to determine the LOD in toxicology: the statistical approach and the empirical approach.[24] The statistical approach requires analysis of ten replicates of a known negative sample, measurement of the system noise, and calculation of a concentration corresponding to three times the matrix noise level. The empirical approach requires analysis of a series of decreasing concentrations of drug in the biological matrix. The LOD is the concentration at which it is no longer possible to detect and identify the drug.

Other methods have included the minimum detectable limit,[22] and probability of detection.[23] For qualitative methods, information theory has been applied to estimate a probability of identification.[25,26] In general, methods to determine the probability of identification require more extensive studies because nonparametric statistical approaches are used.

The LOD should be verified every twelve months or when major maintenance is performed.

6.5.1 Recommendations for Threshold Compounds

The empirical method is recommended for determining the LOD. Serial dilutions of a sample with a known concentration should be made using an appropriate biological matrix as diluent. Given the role of the matrix "noise" in detection, more than one biological matrix diluent may be desirable. The LOD is the lowest concentration where both detection and identification criteria are met. The LOD should be verified by triplicate analysis of a sample where all replicates meet criteria for detection and identification. Any bias between the concentration measured and that expected should not be considered.

6.5.2 Specific Recommendations for Nonthreshold Compounds

The empirical method may be applied to estimate the LOD for nonthreshold compounds that are not quantified. In the case where no reference standard is available, a sample for which the concentration has been estimated may be used. The procedure should clearly state the conditions under which the LOD was determined.

6.6 Limit of Quantitation

The limit of quantitation (LOQ) is the lowest amount of analyte in a sample that can be quantitatively detected with a stated acceptable uncertainty and bias under stated experimental conditions. For GC/MS confirmation analyses, the concentration measured in the appropriate matrix to which a suitable reference material has been added should be within ±20% of the expected value (bias) with a coefficient of variation not greater than 20%. The imprecision of the analysis should be determined using a minimum of three measurements.

The LOQ should be verified every twelve months or when major maintenance is performed.

6.7 Robustness

Robustness is the reliability of an analysis with respect to variations in method parameters. If the results are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. A set of system suitability parameters (e.g., resolution test, minimum signal or signal-to-noise ratio) should be developed to ensure that the validity of the analytical procedure is maintained whenever used (see Section 7).

Typical GC/MS procedure parameters to be evaluated in determining robustness may include:

- stability of analytical solutions;
- hydrolysis conditions (e.g., solutions, temperatures);
- different lots of extraction materials;
- derivatization conditions (e.g., solutions, temperatures);
- stability of analytes after derivatization;
- injection parameters (e.g., volume, temperature, flows);
- different instruments;
- different columns (different lots and/or suppliers);
- temperature (isothermal or programmed) variation;
- flow rate variation; and
- different analysts.

7 Routine Instrument and Method Performance Verification

A GC/MS confirmation method should contain, or refer to, a procedure for documenting proper performance of the analytical instrumentation prior to analyzing any samples. In addition to verifying instrument function daily, quality control samples should be included with each batch of samples to ensure method performance. The selection of the quality control sample should reflect the intended purpose of the analysis.

7.1 Gas Chromatograph

Capillary column gas chromatography is used for the majority of separations used for GC/MS confirmation work. The primary criteria for suitable chromatographic performance are retention time reproducibility, peak narrowness and asymmetry, peak resolution, and signal-to-noise ratio (or signal) at a specified concentration. It is important to note that the peak criteria reflect injector, column, and detector performance at the time of the confirmation. The compounds selected for use in evaluation of the system should be suitable for that purpose. The chromatographic system performance verification mixture should be run at least once each day and must be run after a system repair or modification prior to analysis of any specimen.

Prior to analyzing samples, the chromatographic system should be evaluated for adequate performance. Peak narrowness and asymmetry are good measures of the efficiency of the chromatographic system. The performance verification mixture should include an appropriate compound for which these parameters are monitored. Peak narrowness should be measured by the plate number measured at the half-height of the peak profile. Minimum acceptable values for these criteria must be included in the quality assurance portion of the procedure.

Peak asymmetry is generally measured as the ratio of the distance from a perpendicular dropped from the peak apex to the peak profile at a point 10% of the distance from the baseline to the peak apex. A perfectly symmetrical peak has an asymmetry value of 1.0. A peak for which the distance to the trailing edge is longer than that to the leading edge has a value greater than one and is called "tailed." A peak for which the distance to the leading edge is longer than that to the trailing edge has a value less than one and is called "fronted." The procedure must define an acceptable range of values for peak asymmetry.

A pair of appropriate compounds should be included in the performance verification mixture that assesses the ability of the system to clearly separate the compounds. The test compounds should represent the type of compounds that are being separated in the analysis. The procedure should define an acceptable range of values for resolution. The resolution may be expressed either as the defined calculation for resolution (R_s) or as the depth of valley between the peak apices. For the purposes of this discussion, the valley point height should be measured as a function of the height of the smaller peak. The minimum resolution between an adjacent pair of peaks should be at least 1.25 or approximately 10% valley/peak ratio. The rationale for recommending this chromatographic resolution is that it has been shown that peak height measurements maintain a deviation of less than 1% from the true height (accuracy) at this resolution over a 100-fold ratio of peak sizes. A similar accuracy in peak area can be obtained over a 32-fold range.[27]

System response to a known concentration should also be documented. One or more compounds in the system performance mixture should be evaluated for either absolute signal or signal-to-noise ratio at a specified concentration. The internal standard may also be used for this assessment of system function.

7.2 Mass Spectrometer

7.2.1 Mass Axis and Abundance Calibration

A reference compound, such as perfluorotributylamine (PFTBA), decafluorotriphenylphosphine (DFTPP), or other appropriate compound, must be used to calibrate the mass axis of the mass analyzer. In the case of QMF and ion trap instruments, the reference compound is also used to set the relative abundance of selected ions as part of the tuning process.

7.2.1.1 Acceptance Criteria for the Tuning Compound Perfluorotributylamine

The laboratory must establish acceptable ranges for the relative abundance of ions m/z 69, m/z 219, and m/z 502. Widely accepted specifications for EI QMF instruments are: m/z 69, 70 to 100% of the base peak; m/z 219, greater than 20% of the base peak; and m/z 502, greater than 2% of the base peak. These specifications were developed primarily to minimize the effect of tuning on computerized library spectral matching algorithms. Manufacturers' recommendations should be considered in establishing the relative abundance ranges. Acceptance criteria should also include inspection of the mass peaks for symmetry and the absence of precursors. There should also be resolution between each major ion and its ^{13}C-isotope mass peak. In addition, it is important to monitor the consistency of the ion abundance and instrument settings between days. If an unacceptable change is observed, the underlying reason for such a change should be determined, and the problem corrected. All corrective actions must be documented.

Target tuning for high-mass compounds or selected ion monitoring (SIM) operation is acceptable. Although this approach to tuning precludes interlaboratory comparisons of resulting spectral data, the

increase in sensitivity is usually advantageous. Methodological considerations may require that the m/z 502 ion relative abundance be as high as 50%.

When tuning tandem mass spectrometry instrumentation, the manufacturer's recommendations should be considered.

7.2.1.2 Acceptance Criteria for Alternative Tuning Compounds

Alternative tuning compounds may be used for specific purposes, such as performance enhancements for measurements associated with high molecular weight compounds, for negative ion detection, or for advanced modes of operation such as tandem mass spectrometry (MS/MS). The laboratory must establish acceptable ranges for the relative abundance of ions based on criteria in the peer-reviewed literature or by the instrument manufacturer.

7.2.2 Air Leak Criteria

Proper operation of the mass spectrometer also requires that minimal amounts of air (and water) are contained in the system. The presence of large amounts of air from a leak can influence ionization efficiency, mass resolution, and system sensitivity. The laboratory must establish acceptance criteria for the presence of these compounds.

The recommended approach is to monitor the ions produced from the constituents of air itself. In a leak-free system which has been under vacuum for some time, nearly all the components of air will be pumped away, but water will be observed at m/z 17 and 18 due to the difference in the pumping efficiency for water. If the abundance ratio of m/z 28 (N_2^+) to m/z 18 (H_2O^+) is less than 0.5, the system is free of leaks. If the ratio of m/z 28 to m/z 18 is greater than 2 to 1, there is an air leak. In the case of a leak, the ratio of m/z 28 to m/z 32 (O_2^+) will also be about 3, reflecting the composition of air. Under these circumstances, the leak should be located, fixed, and the repair documented. If the ratio of m/z 28 to m/z 18 is between 0.5 and 2, the situation should be monitored. Note that a large leak may result in saturation of the m/z 28 peak and give falsely low ratios. A noticeable increase in system pressure as well as the presence of significant amounts of m/z 14 (N^+) and m/z 16 (O^+) relative to m/z 18 are indications of a severe air leak.

Some manufacturers have elected to use the abundance of the m/z 28 ion from N_2 relative to m/z 69 from PFTBA tuning reference compound as an indication of an air leak. This approach is based on a consistent flow of PFTBA from the calibration gas vial. It is frequently specified in the range of 10 to 20%. The laboratory may choose to follow the manufacturer's recommendation on the specific relative abundance of these ions as a daily monitor for an air leak.

7.3 Calibration

7.3.1 Procedures

7.3.1.1 Single-Point Calibration

If the purpose of the analysis is to demonstrate that the concentration of a substance is greater than a threshold value, a single calibrator analyzed contemporaneously with a concentration equal to the threshold value may be used. Since the purpose of the measurement is to convey a degree of confidence that the value is greater than the threshold, it is recommended that the margin by which the sample exceeds the threshold be documented to have statistical significance.

7.3.1.2 Multipoint Calibration Curve

When a linear calibration curve is observed, the use of appropriate standard concentrations can maximize the precision of the curve at the threshold concentration. The narrowest point for the confidence band about a linear regression line occurs at the mean value of the ordinate (x) values. Where practical, concentrations for standards should be chosen so that the threshold concentration is at or near the mean value of the concentrations analyzed for the calibration.

The concentrations of the standards should be computed from the linear regression line and should agree within ±20% of the nominal value. If more than three concentrations are used to establish the curve, one standard may be dropped for not meeting acceptance criteria. Standards may not be dropped to improve the curve fit, to bring control results into acceptance range, or simply because its concentration is outside the target range. There must be a logical reason for dropping a standard (e.g., poor recovery, interfering peak, incorrect integration) and it must be documented.

7.3.1.3 Historical Multipoint Calibration Curve

If the standard curve has been documented to be linear and stable over a specified time interval, a historical calibration curve may be used to determine the concentration. In this case, at least two controls or standards should be analyzed (one of which should be at the threshold concentration), and the concentrations and ion ratios must be within ±20% of the initial measurements.

7.3.2 Internal Standard

It is advisable to use an internal standard, added prior to any extraction steps, to assess the performance of sample preparation and instrument function. For mass spectrometric detection, the use of a compound labeled with a stable isotope such as deuterium is recommended. If a deuterated internal standard is not available, or if a number of compounds are confirmed in the same procedure, it is acceptable to use a structurally related compound. The internal standard must undergo any derivatization reactions used in the procedure.

7.4 Quality Control/Quality Assurance

An important component of assuring analytical quality is an active quality assurance program. The program should encompass the range of compounds to be expected in the analysis, although the number of compounds confirmed and the number of analytical batches analyzed may preclude frequent assessment of every compound. The quality control samples must constitute at least 10% of the samples run in an analytical batch. Blind quality control samples should be included in the program which challenge not only the analytical portion of the assay, but also clerical and urine integrity testing if this is included as a part of the confirmation analysis.

Specific criteria must be established for acceptance or rejection of the quality assurance specimens. For quantitative assays, a comprehensive set of criteria, such as Westgard's rules, should be used if the volume of sample batches is sufficient to establish the precision characteristics of the assay. An acceptable alternative is to use ±20% of an established mean. Procedures should state the actions to be taken if quality assurance specimens fail to meet established criteria. Regular, active evaluation of quality assurance results must be documented.

Negative control samples should be routinely run, and must be negative in order to accept analytical batch results. It is recommended that a negative control sample be run immediately before each presumptively positive sample to ensure that there is no carryover from standards or controls.

If the confirmation procedure requires hydrolysis to remove glucuronide, sulfate, glutathione, or other conjugates, the hydrolysis procedure should be routinely assessed for completeness. It is recommended that the hydrolysis control, where available, be included with each analytical batch.

7.4.1 Specific Recommendations for Threshold Compounds

Quality assurance samples must be used near the analytical threshold. The concentration should be such that a positive finding is always achieved. A concentration 20 to 25% above the threshold is frequently used to achieve this goal. The positive control should be used for each threshold if more than one threshold is used in a single assay. A negative control with a concentration 20 to 25% below the threshold may be used to document the ability to determine a negative result near the threshold value. In cases where quantitative results are provided, a control sample may be used to document the linear range of the assay.

7.4.2 Specific Recommendations for Nonthreshold Compounds

Positive and negative control materials should be used in each analytical batch to document the presence and absence of the compound to be identified. It is recommended that the positive control contain a concentration comparable to that of the sample.

8 Identification Principles

In confirmation analyses, the objective is to identify the compound within a determined level of confidence. It has been suggested that the level of confidence for legal proceedings be 1 in 10,000 to 1 in 100,000.[28] Analysis procedures or combinations of procedures with greater information content increase the level of confidence in an identification.

8.1 Gas Chromatograph

Due to its high-peak resolution capacity, the use of capillary gas chromatography in conjunction with mass spectrometry significantly improves the information content of the analysis. It should be noted that co-elution of a peak with a compound of known structure does not prove identity, but rather demonstrates consistency with the identity of the known compound. The retention time of the peak due to the presumed compound should elute within ±1% or ±0.2 minutes (whichever is smaller) of the retention time of a peak of a contemporaneously analyzed standard.

Chromatographic peak overload of matrix components or analytes may cause a shift in retention time. If a deuterated internal standard is used, the analysis may be accepted if the retention time difference between the internal standard and the proposed analyte is the same as the difference observed for a control or standard not exhibiting the peak overload condition. If no deuterated internal standard is used, standard addition of the suspected compound may be used to document co-elution. In this approach, an amount of pure standard comparable to that in the original analysis is added to the sample, and the sample is analyzed using the normal procedure. To document identity, only the chromatographic peak presumptively identified should be increased, and the peak profile at half-height should be within ±10% of the original peak width.

The peak of interest should be separated from any other peaks so that there is resolution of at least 1.25 or a valley of at least 90% between peak maxima (for equal-sized peaks). In GC/MS, the additional information obtained from mass-selective detection can be used to enhance chromatographic resolution. Deconvolution or peak purity algorithms can be used to resolve partially co-eluting peaks.[29] This can also be done manually by inspecting the consistency of mass spectra across the peak profile.

8.2 Mass Spectral Identification

A mass spectrum of an unknown component may provide a definitive identification. For this reason, application of GC/MS under appropriate conditions is considered the "gold standard" for identification. As mentioned above, acquisition of a complete mass spectrum is preferable to acquisition of selected ions. In those cases where high-quality full or partial spectra cannot be obtained, selected ion monitoring is acceptable.

8.2.1 Full-Scan Acquisition

The mass spectrum should be acquired from m/z 40 to at least 100 mass units above the expected molecular mass ion. In applications where ions from the derivatizing reagent dominate the spectrum, it is acceptable to acquire data from just above the mass of the derivatizing reagent. The acquisition of partial spectra may also be acceptable. In this case, the partial spectrum should be compared to that of a contemporaneously analyzed standard. Use of a spectral matching algorithm to compare a partial spectrum to a full spectrum is not acceptable.

Either the absence or presence of ions at a particular mass is informative. If manual inspection and identification of compounds is performed, the laboratory must develop guidelines for comparability of an unknown spectrum to that of a contemporaneously analyzed standard. Some typical guidelines may include: presence in the unknown spectrum of all mass ions with a relative abundance greater than 15% in the reference spectrum; agreement of ion intensities within ±20% relative abundance; absence of ions beyond the molecular ion cluster giving rise to a significant ion in spectrum (e.g., M-90 for TMS); absence of any ions greater than 50% relative abundance in the unknown spectrum that do not occur in the standard spectrum; relative abundance of isotopes in molecular ion cluster consistent with contemporaneous reference material or theory; and the absence of illogical mass losses in the spectrum. Inspection of the consistency of spectra across the peak profile may be helpful. The exercise of scientific expertise and judgment is appropriate.

8.2.2 Computer-Based Spectral Library Matching

The library search mode used in most laboratories is the identification search or reverse search. The search algorithm is designed to assess the presence of a target compound from a database of spectra in a spectrum obtained from a chromatographic peak. It is assumed in these algorithms that ions not in the reference spectrum are from impurities, and these ions are ignored. Other factors may also be applied to the experimental spectra in order to improve the ability of the algorithm to match a spectrum in the library database. The two most common commercially available algorithms are the probability-based matching (PBM) approach[30] and the dot product approach.[b] [31]

The reliability of computer-aided mass spectral matching to a library spectrum is dependent on a number of factors including: the search algorithm; the quality of the experimental spectrum; the presence of the spectrum in the library; the quality of the library spectra; the use of complete versus condensed library spectra; and instrumental factors such as tuning and source temperature. Decreasing analyte concentration makes all ions less abundant and more variable, decreasing the quality of the spectrum and the confidence index for the match.[30]

The laboratory must establish criteria for acceptance of compound identification based on the "spectral match" quality. A spectral match factor of greater than 95 is generally associated with a correct identification. Since the match factor does not guarantee identification, all spectral library matches should

[b] Distributed by the National Institute of Standards and Technology (NIST) and others.

be reviewed by a qualified scientist. It is recommended that for spectral match quality of between 75 and 95, the spectra should be evaluated by a qualified scientist for potential compound identification.

8.2.3 Selected Ion Monitoring Acquisition

As mentioned above, it has been demonstrated that the relative ion abundance ratios of three or four ions can be used to identify a compound. It should be noted that the ions must be diagnostic of the structure of the compound. In general, ions of higher abundance are selected due to their better reproducibility and lower limit of detection. Structurally significant ions should be selected over ions that have greater abundance but are not diagnostic. If sufficiently abundant, one of the ions selected should be the molecular ion (M^+). In any case, not more than one ion should be from any derivatization moiety.

There has been some confusion in the literature about the agreement between the observed ion ratios due to the fact that both the relative abundance and the variation in observed ratios have been described in percentage. At high relative abundance, this is of little consequence. For low relative abundance ions, there is a significant difference in outcome. For example, if a ±20% variation is computed at a relative abundance of 80%, a range of observed relative abundances between 64% and 96% would be accepted. The same criteria applied to an ion of relative abundance 10% results in an acceptable range of only 8 to 12%. If a ±10% abundance criteria is adopted, the corresponding ranges are 70 to 90% and 0 to 20%.

The laboratory must define what its identification criteria are for ion ratio matching. For the unknown compound, two ion ratios (three ions monitored) must be within the acceptable range for identification. The ion of largest abundance is frequently used for quantitative purposes and is referred to as the “quantification or quant ion” while the other two ions monitored for identification purposes are referred to as “qualifier or qual ions." For the internal standard, one ion ratio must be within the acceptable range (two ions monitored). It is recommended that the unknown compound have ion ratios within ±20% variation of the ion ratios measured in a contemporaneously analyzed standard if the relative abundance is greater than 20%, but a ±5% abundance range should be accepted when the measured relative abundance is less than 20% (e.g., 60 ± 12% and 18 ± 5% relative abundance). In addition, if the calculated acceptance range includes zero or a negative relative abundance, the lower end of the acceptance range should be 1% (i.e., the ion must be present).

The ion intensities for identification may be obtained from integrated peak height or peak area ratio measurements or from the ratios within a single acquisition. The ratio may be computed directly from the heights or areas. In order to be consistent with identity, all ions from the same peak profile must appear within one mass spectral acquisition of each other at the apex. If more than one standard is analyzed contemporaneously, the ion ratio acceptance range may be computed from a single standard, an average of standards, or a weighted average of standards. The method of computation must be documented. It is generally not acceptable to use different standards or different methods of computation for different specimens in the batch.

If screening procedure is also GC or GC/MS, and if less than three structurally characteristic ions are available, it is recommended that a second chemical technique or method be used. A change in derivatization chemistry can be considered a different technique.

8.2.4 Chemical Ionization

Chemical ionization mass spectra are characterized by less fragmentation but greater sensitivity than electron ionization. Since the ionization process is based on the kinetics of chemical reactions, the reproducibility of ion-relative abundances is somewhat smaller than for electron ionization. The specificity is dependent on the ionization conditions used and the uniqueness of the ions monitored. The ions produced from chemical ionization can be acquired in either the scan or SIM mode.

Acceptance criteria for compound identification with chemical ionization must be documented. The ion derived from the intact molecule [e.g., $(M+H)^+$ or $(M-H)^-$] or an ion closely related to the molecular species (e.g., loss of HF) should be monitored. Ion ratios compared to a standard run in the same batch should be within ±25% variation (e.g., for 80% relative abundance, acceptance range is 60 to 100%). Computation and evaluation of the ion ratios should be performed as described in Section 8.2.3. If full-scan acquisition is used, there should be no major ions present in the scan that are not derived from the proposed analyte.

8.2.5 Tandem Mass Spectrometry (MS/MS)

Tandem mass spectrometric methods may employ either electron or chemical ionization techniques. The fragmentation process is a physicochemical interaction, and as a result the ion intensity reproducibility is lower than that observed for electron ionization. The selective fragmentation of an ion of defined mass significantly increases the specificity of the technique. The ions produced from collision-induced fragmentation can be acquired in either the scan or SIM mode.

Acceptance criteria for compound identification with MS/MS must be documented. Collision conditions should be selected to ensure that the precursor ion is present in MS/MS scan. When monitoring one precursor to get one product ion, the resolution for the first mass analyzer should be set to unity (i.e. mass window equals one amu). If multiple ions are monitored for identification, ion ratios compared to a standard run in the same batch should be within ±25% variation (e.g., for 80% relative abundance, acceptance range is 60 to 100%). Computation and evaluation of the ion ratios should be performed as described in Section 8.2.3. If full-scan acquisition is used, there should be no major ions present in the scan that are not derived from the proposed analyte.

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NCCLS consensus procedures include an appeals process that is described in detail in Section 9 of the Administrative Procedures. For further information, contact the Executive Offices or visit our website at www.nccls.org.

Summary of Comments and Subcommittee Responses

C43-P: *Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Proposed Guideline*

General

1. Consider including several figures that illustrate both a typical ion chromatograph and a total ion chromatograph.

- **A figure has been placed at the end of Section 4.4.**

2. Overall I'd like to say the primary objective stated as to establish uniform practices for producing quality data for quantitation and identification of drug/metabolites was not met. To a practicing scientist, I don't believe that the explanations were specific enough. While most people have the impression that the SAMHSA guidelines are too strict and dictatorial, there is some comfort in knowing that interpretations are reproducible among labs following these criteria for their drug testing. When I read the title to this guideline, my first thought was great someone is finally going to set some standards in clinical and forensic medicine. However, I think specific examples for how to use these criteria in the clinical and forensic arenas are lacking.

- **The SAMHSA guidelines are written in response to experience with the analysis of five compounds using primarily selected ion monitoring. It would be extremely difficult to cite examples for all of the possible scenarios for clinical and forensic testing. The commenter's opinion that the primary objective is not met, while appreciated, is not shared by the subcommittee**.

Foreword

3. Second paragraph, last sentence, "Appropriate application of these analytical tools requires that the methods are fit for their purpose and the instruments are operating correctly." This is somewhat vague. Are all the preceding methods mentioned in the context of being confirmatory methods? If so, perhaps the sentence should read 'Methods used are fit for the purpose of confirmation and....'

- **The subcommittee believes that the sentence as written describes the situation accurately. Since the entire guideline applies only to confirmation analyses, the specifics of fit for purpose are determined by whether the compound must be quantified or identified.**

Introduction

4. In the Introduction, the statement "establish broader criteria" doesn't specifically say who the comparator is although it is inferred in the abstract that it is the SAMHSA guidelines.

- **The wording has been changed to reflect that the criteria apply to areas other than drugs of abuse testing**.

Section 4

5. There are 5 pages of GC/MS instrument description. Scientists who are looking for guidelines involving GC/MS are probably quite familiar with the technique.

- **While this may be true, the experience of the subcommittee members is that significant amounts of incorrect information are circulated in the workplace. It is also likely that some scientists are not familiar with all of the techniques discussed. The subcommittee believes, therefore, that inclusion of this information establishes a uniform base upon which to build a guideline.**

Section 4.3.4

6. Section 4.3.4 touches on large amounts of air in the system. Section 7.2.2 helps identify ways to assess the system for air but nowhere is it included what instrument parts and parameters are damaged by air leaks.

- **There is specific reference to the damaging effects of air on the filament and column in Section 4.3.4. The deleterious effects on tuning are also indicated. The subcommittee therefore believes that this comment has been addressed.**

Section 6.1.1

7. For non-threshold compounds recommendation, should this include reference to whether these reference materials and metabolite isolates be run at the time of the unknown or run and stored as a database with which to compare the unknown spectrum? On the last line, it is unclear as to whether comparison of your unknown spectra or comparison of your reference materials to a reputable scientific journal is addressed here.

- **A sentence has been added to Section 6.1.1 to indicate that the secondary reference material should be analyzed contemporaneously with the unknown. It seems clear that the spectra obtained from the secondary reference material should be comparable to a spectrum published in the literature.**

Section 6.2

8. Second Paragraph: In this section on accuracy, it may be appropriate to reference CLIA '88 as one suggested interval for calibration verification to assess accuracy: according to CLIA it ‘should be performed at least every 6 months and after major maintenance or change in reagents.’

- **A recommendation of at least every twelve months and after major maintenance has been made in Section 6.2**.

Section 6.2.1

9. Since carryover and contamination are the major sources of false positivity and inaccuracy in any high sensitivity measurements, the committee might consider strengthening Section 6.2.1 by including descriptions or examples of how to avoid or detect carryovers (e.g., negative runs…).

- **A sentence has been added to Section 6.2.1 using a blank injection as an example of a method to detect carryover.**

Section 6.4

10. Linearity, top paragraph. The discussion concerns choice of calibration concentrations for best precision about the regression line, rather than linearity per se. Perhaps a section should be added for calibration, or the heading for Section 6.4 could be changed to 'Calibration' with this paragraph moved first to precede discussion of linearity.

- **There is already a section on Calibration (Section 7.3). The subcommittee concludes that the inclusion of information on the assessment of linearity is best presented here.**

11. Linearity, first paragraph. If peak heights or areas (or ratios if an IS is used) are plotted as a function of analyte concentration to determine assay linearity, it should be clear that the approach to assess linearity must be the same as that used to obtain patient results.

- **Since the remainder of the section discusses the use of linear calibration to establish concentrations, this is implicit if not explicit in the discussion.**

12. Linearity, second paragraph. Correlation coefficient is a particularly poor way to assess assay linearity. It is not sensitive to deviations from linearity and the cut-off of 0.99 has no scientific basis. Reference should be made to NCCLS document EP6. For a discussion of the faults in using correlation coefficient, see Lipman and Astles, Clin Chim Acta, (1999) 282:15-34.

- **A revised paragraph discussing the limitations of correlation coefficient in determining linearity has been incorporated along with several additional references. A reference to NCCLS document EP6 was also added at the end of the section.**

13. Linearity, third paragraph. Authors may want to make the recommended frequency for checking linearity (currently yearly) to be the same as the frequency for checking calibration verification (Section 6.2).

- **The recommendation for linearity check has been changed to every twelve months.**

Section 6.5

14. It would be pertinent to include standard ways in which the industry is verifying LOD annually. Also, what specificity criteria should be applied to determining LOD for non-threshold compounds?

- **The third paragraph deals specifically with the current industry standard for verifying LOD.**

Section 6.6

15. It would be pertinent to include standard ways in which the industry is verifying LOQ annually.

- **The section deals specifically with the current industry standard for verifying LOQ.**

Section 7

16. Routine Instrument and Method Performance, second sentence. A statement is made that 'quality assurance samples' should be included with each batch. 'Quality control samples' would be more appropriate and that phrasing is used elsewhere (Section 7.4, first paragraph)."

- **The wording has been changed to "quality control samples."**

Section 7.1

17. Most people using GC do not even know what theoretical plates are let alone how to calculate them. It seems inappropriate to say they should calculate something we all know they probably will not. Perhaps something that they will actually do would be better.

    The measurement of peak width at half height is something they can reasonably do. I think it should say something about ensuring the peak is represented in such a way that this measurement is possible. I have seen some peaks that are drawn on a wide time scale that the peak looks like nothing more than a spike, making the measurement impossible. Also, saying that time is acceptable to measure is reasonable, but distance seems inappropriate. Since the representation of a peak can be changed by telling the system how to draw it, as a result, distance can be satisfied by any peak. Measuring distance to determine asymmetry is reasonable. Perhaps I am not clear on what is meant by this paragraph.

- **Guidelines are intended to reflect good practice. If they can measure the peak width at half height, they can calculate theoretical plates.**

  The purpose of measuring theoretical plates is twofold. First, since the width of the peak changes with the retention time (for an isothermal system), absolute width is not a good measure of anything. Second, since theoretical plates is a dimensionless ratio, the peak width and retention time can be measured in any units, so long as they are on the same scale (not expanding the scale for one and not the other). A definition of plate number has been included in Section 5 to assist in calculations.

18. Regarding the statement that "the rationale for recommendation of this resolution is the deviation is < 1% for…." Please reference this citation.

- **A reference has been added.**

Section 7.3

19. Section 7.3 doesn't address the differences in matrix found within the clinical and forensic practices.

- **A wide variety of matrices are found within clinical and forensic practice, and discussion of all of them is beyond the scope of this document.**

Section 7.4

20. Obtaining blind QC in these matrices presents the same problem. This also does not address how to make, how to store, and how to use these so-called quality control samples which should comprise 10% of an analytical batch.

- **The production of quality control materials is beyond the scope of this guideline. While the topic is of great interest, the strategies and limitations required for production of control materials in various matrices would require a separate document.**

Section 7.4.2

21. This might necessitate creation of a QC material with each confirmation run. While this practice is probably okay, there should be documentation that at some point the material used to create this QC or metabolite isolate was verified as to purity, etc. This criterion also suggests concentration matching

of a nonthreshold compound for which quantitation criteria have not been established and with no suggestion for how one might concentration match.

- **The production of quality control materials is beyond the scope of this guideline. While the topic is of great interest, the strategies and limitations required for production of control materials in various matrices would require a separate document.**

Section 7.4

22. It is not clear to me how a conjugated internal standard can be used to assess hydrolysis of a sample. This should be explained or I think it is something that people will not get right.

- **The subcommittee agrees and the last sentence of Section 7.4 has been deleted.**

Section 8.1

23. Most drug labs currently use ±2% for time. Is there any reason to set this to 1%? I have no real objection except we need to realize that this would make many labs change criteria. I do have serious concerns about using absolute retention time for differences. ±0.2 minutes seems a problem for some long runs and unreasonably long for short retention time, particularly with the movement to shorter run times. As some drug testing labs work to get drug retention times down to 1-2 minutes, that criterion translates to ± 10-20%.

- **The purpose of this guideline is to address a broad spectrum of confirmation activities, not just drugs of abuse testing which is a high volume subset of the testing world. It would be appropriate to keep this broader spectrum of use in mind when discussing the guideline.**

  That said, this is certainly a difficult issue. A ±2% interval at 20-minute retention time is ±0.4 minutes; a 1% interval is ±0.2 minutes which should be achievable. The subcommittee agreed that it should be possible to achieve 1% reproducibility in retention time. The phrase "whichever is smaller" was added to the first paragraph.

24. A valley of 20% is too high. Typically 10% is the most allowed. Why 20%? Also, there appears to be a discrepancy between this section and section 7.1. Also, it defines it as a valley assuming equal sized peaks, which seldom happens. There should be something said about unequal sized peaks, particularly when assessing the valley based on the peak of interest, not the largest (which many labs want to do).

- **The guideline now states that a resolution of 1.25 is required, which by definition is a 10% valley for equal sized peaks. The valley for a resolution of 1.25 changes with relative peak size, and for whether the peak of interest is the larger or the smaller peak. The point is that the use of a valley calculation is not recommended due to the changing situation with relative peak height. Additional discussion of the resolution issue is included in Section 7.1.**

Section 8.2.2

25. This section should give some guidelines about subtraction of background; not necessarily how to, but at least some things that are not appropriate.

- **The subcommittee believes this is beyond the scope of the document because there is not a standard approach which is varied between software programs.**

Section 8.2.3

26. At the end of the second to last paragraph discussion of not using different standards or different methods of computation for different specimens in a batch is discussed. While this is ideal, in the interest of time and cost, more than one presumptive positive may be included in a confirmation run. The article already discusses ways in which the concentration of a compound affects its identification and quantitation and adjustments that can be made to relative abundance and abundance criteria. I will say from experience that this is almost the rule and not the exception that specimens of differing concentrations, differing metabolites and differing ways in which individual bodies produce metabolites will be in the same batch confirmation run and will need essentially different identification criteria placed on them to determine positivity.

- **The comment, while relevant, does not indicate what the author would like the subcommittee to do.**

27. I get concerned with using absolute ion ratio ranges, particularly at the low end. Although I understand the problem with low abundance results, although my experience says it has more to do with low abundance than low relative abundance if the absolute abundance is high enough. No excuse for absolute ranges for urine drug testing except for a few compounds that are monitored at very low concentrations. Also paragraphs two and three give different suggestions of implementation. The first is particularly troublesome as I have inspected a lab that used absolute ranges that went from 0-20% for one of the PCP ions and guess what? 1% is inadequate in my view because that could well be background noise. To use such a criteria should be reserved only for rare and unique circumstances and not proposed as an option for most analyses.

- **The purpose of this guideline is to address a broad spectrum of confirmation activities, not just drugs of abuse testing, which is a high volume subset of the testing world. It would be appropriate to keep this broader spectrum of use in mind when discussing the guideline.**

  This comment is somewhat difficult to understand, since by definition the mass spectrum is reported in relative abundance; thus the term "absolute abundance" is not clear.

  Outside of the world of drugs of abuse, there are many examples of compounds that yield relatively few ions of relative abundance greater than 10%. Several publications have shown that in this case, the reproducibility of the ion signal from a mass spectrometer may not achieve ±10% — in essence, you are requiring better ion signal reproducibility for low intensity ions than you are for more intense ions.

  The concept of using "0%" abundance for any ion makes little sense. This issue has been addressed in the third paragraph of Section 8.2.3.

Related NCCLS Publications*

EP6-P2 **Evaluation of the Linearity of Quantitative Analytical Methods: A Statistical Approach; Proposed Guideline—Second Edition (2001).** This document provides guidelines for characterizing the linearity of a method during a method evaluation; for checking linearity as part of routine quality assurance; and for determining and stating a manufacturer's claim for linear range.

M29-A2 **Protection of Laboratory Workers from Occupationally Acquired Infections – Second Edition; Approved Guideline (2001)**. This document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of bloodborne infection from laboratory instruments and materials; and recommendations for the management of bloodborne exposure.

NRSCL8-A **Terminology and Definitions for Use in NCCLS Documents; Approved Standard (1998).** This document provides standard definitions for use in NCCLS standards and guidelines, and for submitting candidate reference methods and materials to the National Reference System for the Clinical Laboratory (NRSCL).

T/DM6-A **Blood Alcohol Testing in the Clinical Laboratory; Approved Guideline (1997).** This document provides technical and administrative guidance on laboratory procedures related to blood alcohol testing, including specimen collection, methods of analysis, quality assurance, and reporting of results.

T/DM8-A **Urine Drug Testing in the Clinical Laboratory; Approved Guideline (1999).** This guideline addresses the development of procedures for analysis of urine to determine the presence of certain controlled substances; for specimen collection and processing; for methods of analysis; for quality assurance; and for the reporting and interpretation of results.

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

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