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Review Article

A Review on GC-MS and Method Development and Validation

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ABSTRACT
Gas chromatography–mass spectrometry (GC-MS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. Analytical methods development and validation play important roles in the discovery, development and Manufacture of pharmaceuticals. Method development is the process of proving that an analytical method is acceptable for use to measure the concentration of an API in a specific compounded dosage form which allow simplified procedures to be employed to verify that an analysis procedure, accurately and consistently will deliver a reliable measurement of an active ingredient in a compounded preparation. The analytical method validation is essential for analytical method development and tested extensively for specificity, linearity, accuracy, precision, range, detection limit, quantization limit, and robustness. In summary, analytical method development and validation allows to confirm that an accurate and reliable potency measurement of a pharmaceutical preparation can be performed.

Key words:

INTRODUCTION

Gas chromatography–mass spectrometry (GC-MS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample[1]. Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples. GC-MS can also be used in airport security to detect substances in luggage or on human beings. Additionally, it can identify trace elements in materials that were previously thought to have disintegrated beyond identification[2].

GC-MS has been widely heralded as a "gold standard" for forensic substance identification because it is used to perform a *specific test*. A specific test positively identifies the actual presence of a particular substance in a given sample. A *non-specific test* merely indicates that a substance falls into a category of substances. Although a non-specific test could statistically suggest the identity of the substance, this could lead to false positive identification[3].

History: The use of a mass spectrometer as the detector in gas chromatography was developed during the 1950s after being originated by James and Martin in 1952. These sensitive devices were originally limited to laboratory settings[4].

In 1964, Electronic Associates, Inc. (EAI), a leading U.S. supplier of analog computers, began development of a computer controlled quadrupole mass spectrometer[2] under the direction of Robert E. Finnigan. By 1966 Finnigan and collaborator Mike Uthe's EAI division had sold over 500 quadrupole residual gas-analyzer instruments[5]. In 1967, the Finnigan Instrument Corporation was formed and in early 1968, delivered the first prototype quadrupole GC/MS instruments to Stanford and Purdue University.

In 1996 the high-speed GC-MS units completed analysis of fire accelerants in less than 90 seconds, whereas first-generation GC-MS would have required at least 16 minutes. By the 2000s computerized GC/MS instruments using quadrupole technology had become both essential to chemical research and one of the foremost instruments used for organic analysis[6]. Today computerized GC/MS instruments are widely used in environmental monitoring of water, air, and soil; in the regulation of agriculture and food safety and in the discovery and production of medicine[7].

This guidance provides general recommendations for bioanalytical method validation. The recommendations can be adjusted or modified depending on the specific type of analytical method used.

Instrumentation: The GC-MS is composed of two major building blocks: the gas chromatograph and the mass spectrometer. The gas chromatograph utilizes a capillary column which depends on the column's dimensions (length, diameter, film thickness) as well as the phase properties (e.g. 5% phenyl polysiloxane). The difference in the chemical properties between different molecules in a mixture will separate the molecules as the sample travels the length of the column. The molecules are retained by the column and then elute (come off of) from the column at different times (called the retention time), and this allows the mass spectrometer downstream to capture, ionize,

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accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer do this by breaking each molecule into ionized fragments and detect the fragments[8]. GC-MS schematic: These two components, used together, allow a much finer degree of substance identification than used separately. It is not possible to make an accurate identification of a particular molecule by gas chromatography or mass spectrometry alone. The mass spectrometry process normally requires a very pure sample while gas chromatography using a traditional detector (e.g. Flame ionization detector) cannot differentiate between multiple molecules that happen to take the same amount of time to travel through the column (*i.e.* have the same retention time), which results in two or more molecules that co-elute[9]. Sometimes two different molecules can also have a similar pattern of ionized fragments in a mass spectrometer (mass spectrum). Combining the two processes reduces the possibility of error.

Purge and trap GC-MS: For the analysis of volatile compounds a purge and trap (P&T) concentrator system may be used to introduce samples. The target analytes are extracted and mixed with water and introduced into an air tight chamber. An inert gas such as Nitrogen (N_2) is bubbled through the water; this is known as purging. The volatile compounds move into the headspace above the water and are drawn along a pressure gradient (caused by the introduction of the purge gas) out of the chamber. The volatile compounds are drawn along a heated line onto a 'trap'. The trap is a column of adsorbent material at ambient temperature that holds the compounds by returning them to the liquid phase. The trap is then heated and the sample compounds are introduced to the GC-MS column via a volatiles interface, which is a split inlet system. P&T GC-MS is particularly suited to volatile organic compounds (VOCs)[10].

Types of mass spectrometer detectors: The most common type of mass spectrometer (MS) associated with a gas chromatograph (GC) is the quadrupole mass spectrometer, sometimes referred to by the Hewlett-Packard (now Agilent) trade name "Mass Selective Detector" (MSD). Another relatively common detector is the ion trap mass spectrometer. Additionally one may find a magnetic sector mass spectrometer, however these particular instruments are expensive and bulky and not typically found in high-throughput service laboratories. Other detectors may be encountered such as time of flight (TOF), tandem quadrupoles[11].

Ionization: After the molecules travel the length of the column, pass through the transfer line and enter into the mass spectrometer they are ionized by various methods with typically only one method being used at any given time[12]. Once the sample is fragmented it will then be detected, usually by an electron multiplier diode, which essentially turns the ionized mass fragment into an electrical signal that is then detected.

Electron ionization: In the electron ionization (EI) the molecules enter into the MS (the source is a quadrupole or the ion trap itself in an ion trap MS) where they are bombarded with free electrons emitted from a filament, not unlike the filament one would find in a standard light bulb. The electrons bombard the molecules, causing the molecule to fragment in a characteristic and reproducible way. This "hard ionization" technique results in the creation of more fragments of low mass to charge ratio (m/z). Hard ionization is considered by mass spectrometrists as the employ of molecular electron bombardment, whereas "soft ionization" is charge by molecular collision with an introduced gas. The molecular fragmentation pattern is dependant upon the electron energy applied to the system, typically 70 eV (electron Volts)[13]. The use of 70 eV facilitates comparison of generated spectra with library spectra using manufacturer-supplied software or software developed by the National Institute of Standards (NIST-USA).

Cold electron ionization: The "hard ionization" process of electron ionization can be softened by the cooling of the molecules before their ionization, resulting in mass spectra that are richer in information. In this method named cold electron ionization (Cold-EI) the molecules exit the GC column, mixed with added helium make up gas and expand into vacuum through a specially designed supersonic nozzle, forming a supersonic molecular beam (SMB). Collisions with the make up gas at the expanding supersonic jet reduce the internal vibrational (and rotational) energy of the analyte molecules, hence reducing the degree of fragmentation caused by the electrons during the ionization process[14]. Cold-EI mass spectra are characterized by an abundant molecular ion while the usual fragmentation pattern. The enhanced molecular ions increase the identification probabilities of both known and unknown compounds, amplify isomer mass spectral effects and enable the use of isotope abundance analysis for the elucidation of elemental formulae[15].

Chemical ionization: In chemical ionization a reagent gas, typically methane or ammonia is introduced into the mass spectrometer. Depending on the technique (positive CI or negative CI) chosen, this reagent gas will interact with the electrons and analyte and cause a 'soft' ionization of the molecule. A softer ionization fragments the molecule to a lower degree than the hard ionization of EI. One of the main benefits of using chemical ionization is that a mass fragment closely corresponding to the molecular weight of the analyte of interest is produced.

In positive chemical ionization (PCI) the reagent gas interacts with the target molecule, most often with a proton exchange. This produces the species in relatively high amounts[16].

In negative chemical ionization (NCI) the reagent gas decreases the impact of the free electrons on the target analyte. This decreased energy typically leaves the fragment in great supply.

ANALYSIS

A mass spectrometer is typically utilized in one of two ways: full scan or selected ion monitoring (SIM). The typical GC-MS instrument is capable of performing both functions either individually or concomitantly, depending on the setup of the particular instrument.

The primary goal of instrument analysis is to quantify an amount of substance. This is done by comparing the

relative concentrations among the atomic masses in the generated spectrum. Two kinds of analysis are possible, comparative and original. Comparative analysis essentially compares the given spectrum to a spectrum library to see if its characteristics are present for some sample in the library.

Another method of analysis measures the peaks in relation to one another. In this method, the tallest peak is assigned 100% of the value, and the other peaks being assigned proportionate values. All values above 3% are assigned[17].

The total mass of the unknown compound is normally indicated by the parent peak. The isotope pattern in the spectrum, which is unique for elements that have many isotopes, can also be used to identify the various elements present. Once a chemical formula has been matched to the spectrum, the molecular structure and bonding can be identified, and must be consistent with the characteristics recorded by GC-MS. Typically, this identification done automatically by programs in the instrument, given a list of the elements which could be present in the sample.

A "full spectrum" analysis considers all the "peaks" within a spectrum. Conversely, selective ion monitoring (SIM) only monitors selected peaks associated with a specific substance. This is a fast and efficient analysis. When the amount of information collected about the ions in a given gas chromatographic peak decreases, the sensitivity of the analysis increases. So, SIM analysis allows for a smaller quantity of a compound to be detected and measured, but the degree of certainty about the identity of that compound is reduced.

Full scan MS: Full scan is useful in determining unknown compounds in a sample. It provides more information than SIM when it comes to confirming or resolving compounds in a sample. During instrument method development it may be common to first analyze test solutions in full scan mode to determine the retention time and the mass fragment fingerprint before moving to a SIM instrument method.

Selected ion monitoring: In selected ion monitoring (SIM) certain ion fragments are entered into the instrument method and only those mass fragments are detected by the mass spectrometer. The advantages of SIM are that the detection limit is lower since the instrument is only looking at a small number of fragments (e.g. three fragments) during each scan. More scans can take place each second[18]. Since only a few mass fragments of interest are being monitored. It is relatively important to be sure that the ion ratios of the various mass fragments are comparable to a known reference standard.

General uses:

- Identification and quantitation of volatile and semi volatile organic compounds in complex mixtures.
- Determination of molecular weights and elemental compositions of unknown organic compounds in complex mixtures.
- Structural determination of unknown organic compounds in complex mixtures both by matching their spectra with reference spectra and by a prior spectral interpretation.

Applications:

Environmental monitoring and cleanup: GC-MS is becoming the tool of choice for tracking organic pollutants in the environment. The cost of GC-MS equipment has decreased significantly, and the reliability has increased at the same time, which has contributed to its increased adoption in environmental studies. There are some compounds for which GC-MS is not sufficiently sensitive, including certain pesticides and herbicides, but for most organic analysis of environmental samples, including many major classes of pesticides, it is very sensitive and effective.

Criminal forensics: GC-MS can analyze the particles from a human body. The analysis of fire debris using GC-MS is well established, and there is even an established American Society for Testing Materials (ASTM) standard for fire debris analysis.

Law enforcement: GC-MS is increasingly used for detection of illegal narcotics.It is also commonly used in forensic toxicology to find drugs and/or poisons in biological specimens of suspects, victims, or the deceased.

Sports anti-doping analysis: GC-MS is the main tool used in sports anti-doping laboratories to test athletes' urine samples for prohibited performance-enhancing drugs, for example anabolic steroids[19].

Food, beverage and perfume analysis: Foods and beverages contain numerous aromatic compounds, some naturally present in the raw materials and some forming during processing. GC-MS is extensively used for the analysis of these compounds which include esters, fatty acids, alcohols, aldehydes, terpenes etc.

Astrochemistry: Venera 11 and 12 and Pioneer Venus analysed the atmosphere of Venus with GC-MS.

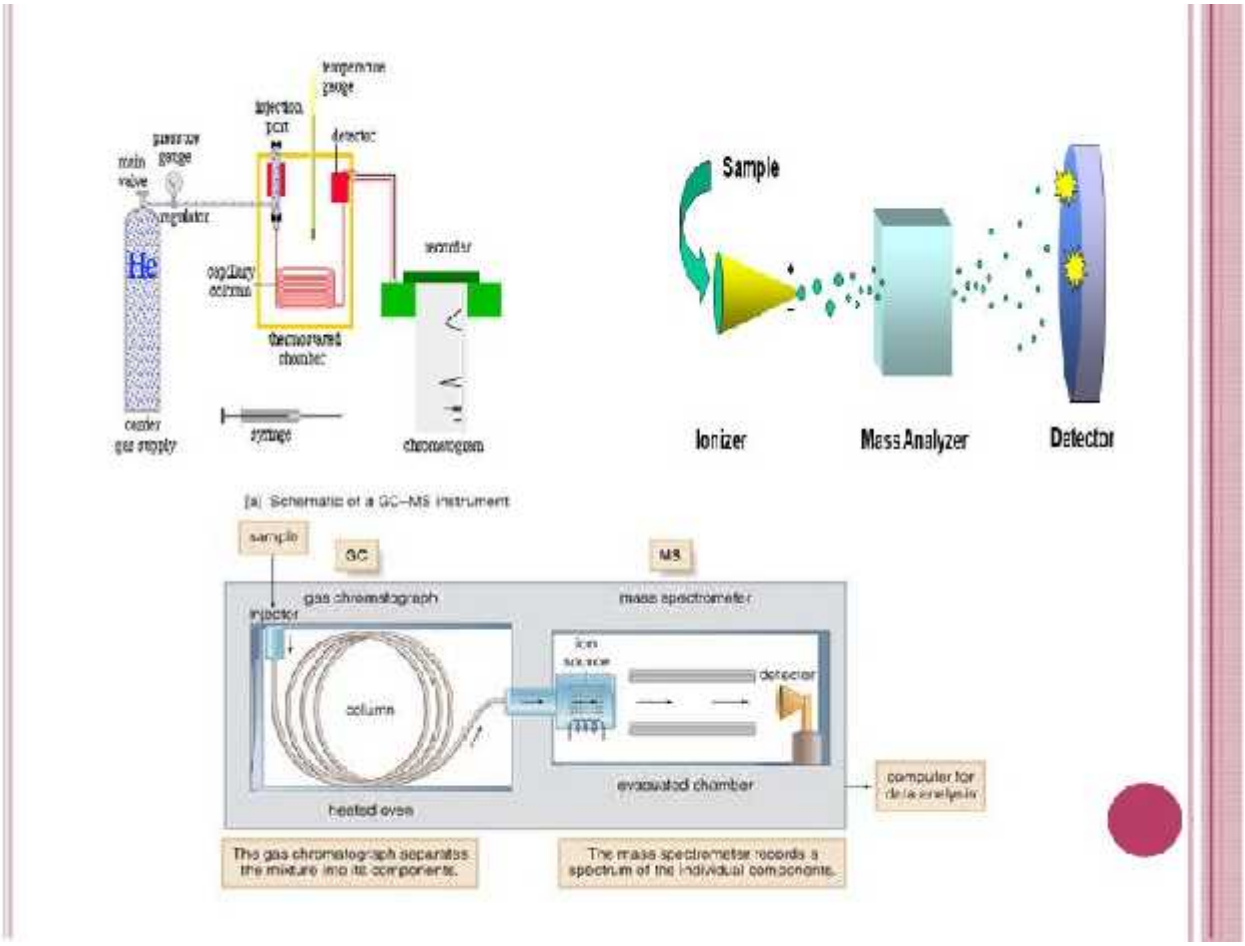
Calibration Procedure/Performance Verification of Instrument And Equipment

Introduction: The performance of laboratory instruments and equipment may change with time, either in the short term owing to fluctuations in the environment or, in the long term, owing to ageing of the mechanical, optical or electronic components. Within a laboratory which maintains a comprehensive quality system, all aspects of analytical work are controlled, and these potential instrumental errors are controlled by carrying out regular preventative maintenance and calibration procedures.

The way in which the performance of instruments and equipment is to be monitored and the frequency of the calibration checks (calibration interval), should be stipulated in Standard Operating Procedures (SOPs).

Performance verification should be based on tests which are not specific to particular methods and which use traceable calibrators and standards, thus allowing equipment to be compared between laboratories. Performance verification is not specifically related to either screening or confirmatory methods. The calibration of instruments and equipment (e.g., wavelength calibration of an IR spectrometer, mass calibration of a GCMS) is independent of the type of sample.

Two conceptual approaches to the calibration process exist:



GC-MS INSTRUMENT FLOW CHART

""The traditional approach, where all instruments and equipment are calibrated and"" The approach where calibration applies only to instruments providing physical measurements, and where the result is a direct measurement of a traceable physical parameter. For example, balances, spectrometers, thermometers, centrifuges and chronometers may be calibrated because there are traceable[21].

Procedures for calibration: Calibration procedures for apparatus used in analytical chemistry are often supplied by the manufacturer, together with information on routine maintenance and the frequency at which these are carried out. The following paragraphs provide guidelines for writing and performing standard calibration procedures for commonly used instruments and equipment.

Gas chromatographs: Routine maintenance operations used include checks on the septum, injector liner, gas pressures and inlet filters (e.g. oxygen scrubber, moisture trap and charcoal trap),baseline signal level and background noise[22]. Depending on the degree of usage of the instrument, it is sensible to have a routine maintenance programme involving weekly change of the septum and injector liner .

Parameter to be calibrated: Oven temperature.

Method: Checked with a portable reference pyrometer or precision thermometer, which should be placed as close as possible to the oven temperature sensor.

Calibration interval: Annually.

Parameters to be verified*:* Column performance (efficiency, resolution, peak shape, retention times).

Method: A set of regularly-used standard(s) is analysed. The precision of retention time(s) can be measured by injecting the standard three times or more. Peak areas can also be measured (see below under integrators). It is useful to plot parameters such as retention times/indices on a control chart.

Verification interval : Monthly.

Parameters to be verified: Detector sensitivity, baseline signal and background noise.

Method: A set of regularly-used standard(s) is analysed and compared with previous runs.

Verification interval: Monthly.

Calibration/performance verification of instruments and equipment

Parameters to be calibrated: Flow rates of detector gases.

Method: A bubble flow meter or calibrated electronic flow meter is used according to the manufacturer's instructions.

Calibration interval: When detector is cleaned or serviced, or the analytical column is changed, or when performance has deteriorated. Difficulty in lighting a flame ionization detector often indicates that the flow rates are incorrect.

Mass spectrometers: Mass spectrometers are tuned and calibrated in a similar manner whether they are stand-alone instruments or combined with chromatographic interfaces

(GC-MS and LC-MS and their multi-sector derivations). Differences arise between quadrupole and magnetic sector instruments, especially if the latter are capable of high resolution[23].

Most bench-top instruments are controlled directly by a computer data system, and tuning and calibration are carried out automatically. Warnings are generated by the data system if the instrument fails to achieve the pre-set performance characteristics, often mandating operator intervention, for example to clean the source.

Parameters to be calibrated: Source tuning and mass calibration.

Method: A calibration compound such as perfluorokerosene (PFK) or heptacosafluorotributylamine (perfluorotributylamine) is introduced to the spectrometer using a direct inlet device. The source is tuned using selected fragment ions to give optimum sensitivity and peak shape, and obtain peak ratios (for example, of m/z 69, 219 and 264 and 502 in the perfluorotributylamine spectrum) usually determined by the manufacturer. Spectra are recorded and compared with the reference spectrum with respect to mass assignments and relative peak intensities.

Calibration interval: Daily or immediately prior to use.

Daily (Continuing) GC/MS Calibration

1. Prior to start of sample analysis; inject 50ng of the bromofluorobenzene tuning compound. Insure that all BFB tune criteria are met according to Table

2. The initial calibration curve for each compound of interest must be checked and verified once every 12 hours of analysis time. This is accomplished by analyzing a calibration standard at the mid-point concentration for the working range of the GC/MS by checking the SPCC and CCC compounds.

3. System Performance Check Compounds (SPCCs): A system performance check must be made every 12 hours. If the SPCC criteria are met, a comparison of response factors is made for all compounds. If the minimum response factors are not met, the system must be evaluated, and corrective action must be taken before sample analysis may begin.

4. Calibration Check Compounds (CCCs): After the system performance check is met, CCCs listed in section.

5. Above are used to check the validity of the initial calibration. If the % Difference (%D) for each CCC is less than or equal to 20%, the initial calibration is considered valid and may be used to quantitate sample data. Calculate the percent difference using:

$$\% \text{ Difference} = \frac{RF_i - RF_c}{RF_i} \times 100$$

ANALYTICAL METHOD DEVELOPMENT

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one, very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias[26]. It becomes necessary, therefore to develop newer analytical methods for such drugs.

Basic criteria for new method development of drug analysis:

- The drug may not be official in any pharmacopoeias.
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations.
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients.
- Analytical methods for the quantification of the drug in biological fluids may not be available.
- Analytical methods for a drug in combination with other drugs may not be available.
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

ANALYTICAL METHOD VALIDATION

Analytical Method Validation is "the collection and evaluation of data, from the process design stage throughout production, which establishes scientific evidence that a process is capable of consistently delivering quality products".

Validation is an act of proving that any procedure, process, equipment, material, activity or system performs as expected under given set of conditions and also give the required accuracy, precision, sensitivity, ruggedness, etc.

When extended to an analytical procedure, depending upon the application, it means that a method works reproducibly, when carried out by same or different persons, in same or different laboratories, using different reagents, different equipments, etc[28].

Advantages of analytical method validation: The biggest advantage of method validation is that it builds a degree of confidence, not only for the developer but also to the user. Although the validation exercise may appear costly and time consuming, it results inexpensive, eliminates frustrating repetitions and leads to better time management in the end. Minor changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process.

Validation parameters:

The various parameters according to the ICH Guidelines as follows[29]

The fundamental parameters for this validation include

(1) accuracy,
(2) precision,
(3) selectivity,
(4) sensitivity,
(5) reproducibility, and

(6) stability.

Different types and levels of validation are defined and characterized as follows:

A. Full Validation

B. Partial Validation

C. Cross-Validation

Validation Procedure for Bioanalytical methods

Selectivity: *Selectivity* is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least 5 six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ).

Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics. If the method is intended to quantify more than one analyte, each analyte should be tested to ensure that there is no interference.

Accuracy: The ***a****ccuracy* of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy.

Precision: The *precision* of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, interbatch precision or repeatability, which measures precision with time, and may involve different analysts, equipment, reagents, and laboratories.

Recovery: The ***recovery*** of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

Calibration/Standard Curve: A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the 6 sample. A sufficient number of standards should be used to adequately define the relationship between concentration and response. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ.

1. Lower Limit of Quantification (LLOQ)

The lowest standard on the calibration curve should be accepted as the limit of

quantification if the following conditions are met:

- The analyte response at the LLOQ should be at least 5 times the response compared to blank response.
- Analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%.

2. Calibration Curve/Standard Curve/Concentration-Response

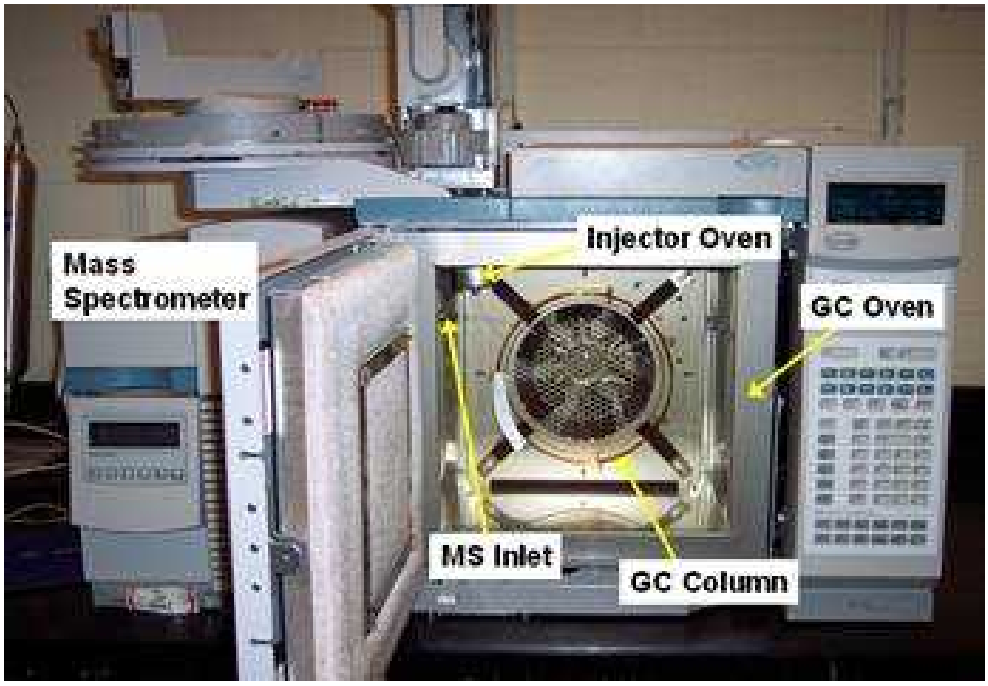
The simplest model that adequately describes the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation shouldmbe justified. The following conditions should be met in developing a calibration curve:

- #20% deviation of the LLOQ from nominal concentration
- #15% deviation of standards other than LLOQ from nominal concentration

At least four out of six non-zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration. Excluding the standards should not change the model used.

Stability: Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability

of the analytes during sample collection and handling, after long-term (frozen at the 7 intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability



GC-MS INSTRUMENT PARTS

experiments should reflect situations likely to be encountered during actual sample handling and analysis.

The procedure should also include an evaluation of analyte stability in stock solution.

All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations.

Specific Recommendations for Method Validation

- The matrix-based standard curve should consist of a minimum of six standard points, excluding blanks, using single or replicate samples. The standard curve should cover the entire range of expected concentrations.
- Standard curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests for *goodness of fit.*
- LLOQ is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision. The LLOQ should be established using at least five samples independent of standards and determining the coefficient of variation and/or appropriate confidence interval. The LLOQ should serve as the lowest concentration on the standard curve and should not be confused with the limit of detection and/or the low QC sample. The highest standard will define the upper limit of quantification (ULOQ) of an analytical method.
- For validation of the bioanalytical method, accuracy and precision should be determined using a minimum of five determinations per concentration level (excluding blank samples).The mean value should be within ±15% of the theoretical value, except at LLOQ, where it should not deviate by more than ±20%. The precision around the mean value should not exceed 15% of the CV, except for LLOQ, where it should not exceed 20% of the CV.Other methods of assessing accuracy and precision that meet these limits may be equally acceptable.
- The accuracy and precision with which known concentrations of analyte in biological matrix can be determined should be demonstrated. This can be accomplished by analysis of replicate sets of analyte samples of known concentrations C QC samples C from an equivalent biological matrix. At a minimum, three concentrations representing the entire range of the standard curve should be studied: one within 3x the lower limit of quantification (LLOQ) (low QC sample), one near the center (middle QC), and one near the upper boundary of the standard curve (high QC).
- Reported method validation data and the determination of accuracy and precision should include all outliers; however, calculations of accuracy and precision excluding values that are statistically determined as outliers can also be reported.
- The stability of the analyte in biological matrix at intended storage temperatures should be established. The influence of freeze-thaw cycles (a minimum of three cycles at two concentrations in triplicate) should be studied. The stability of the analyte in matrix at ambient temperature should be evaluated over a time period equal to the typical sample preparation, sample handling, and analytical run times.
- Reinjection reproducibility should be evaluated to determine if an analytical run could be reanalyzed in the case of instrument failure.
- The specificity of the assay methodology should be established using a minimum of six independent sources of the same matrix. For hyphenated mass spectrometry-based methods, however, testing six independent matrices for interference may not be important. In the case of LC-MS and LC-MS-MS-based procedures, matrix effects should be investigated to ensure that precision, selectivity, and

sensitivity will not be compromised. Method selectivity should be evaluated during method development and throughout method validation and can continue throughout application of the method to actual study samples.

- Acceptance/rejection criteria for spiked, matrix-based calibration standards and validation QC samples should be based on the nominal (theoretical) concentration of analytes. Specific criteria can be set up in advance and achieved for accuracy and precision over the range of the standards, if so desired.

validation documentation/protocol: The validity of an analytical method should be established and verified by laboratory studies, and documentation of successful completion of such studies should be provided in the assay validation report. General and specific SOPs and good record keeping are an essential part of a validated analytical method. The data generated for bioanalytical method establishment and the QCs should be documented and available for data audit and inspection. Documentation for submission to the Agency should include

(1) summary information,

(2) method development and establishment,

(3) bioanalytical reports of the application of any methods to routine sample analysis, and

(4) other information applicable to method development and establishment and/or to routine sample analysis[30].

A. Summary Information: Summary information should include:

- Summary table of validation reports, including analytical method validation, partial revalidation, and cross-validation reports. The table should be in chronological sequence, and include assay method identification code, type of assay, and the reason for the new method or additional validation (e.g., to lower the limit of quantitation).
- Summary table with a list, by protocol, of assay methods used. The protocol number, protocol title, assay type, assay method identification code, and bioanalytic report code should be provided.

B. Documentation for Method Establishment: Documentation for method development and establishment should include:

- An operational description of the analytical method.
- Evidence of purity and identity of drug standards, metabolite standards, and internal standards used in validation experiments.
- A description of stability studies and supporting data.
- A description of experiments conducted to determine accuracy, precision, recovery,selectivity, limit of quantification, calibration curve (equations and weighting functions used, if any), and relevant data obtained from these studies.
- Documentation of intra- and inter-assay precision and accuracy
- Any deviations from SOPs, protocols, or GLPs (if applicable), and justifications for deviations

C. Application to Routine Drug Analysis: Documentation of the application of validated bioanalytical methods to routine drug analysis should include:

- Evidence of purity and identity of drug standards, metabolite standards, and internal standards used during routine analyses
- Summary tables containing information on sample processing and storage. Tables should include sample identification, collection dates, storage prior to shipment, information on shipment batch, and storage prior to analysis. Information should include dates, times, sample condition, and any deviation from protocols.
- Summary tables of analytical runs of clinical or preclinical samples. Information should include assay run identification, date and time of analysis, assay method, analysts, start and stop times, duration, significant equipment and material changes, and any potential issues or deviation from the established method.
- Equations used for back-calculation of results.
- Tables of calibration curve data used in analyzing samples and calibration curve summary data.
- Deviations from the analysis protocol or SOP, with reasons and justifications for the deviations 19.

D. Other Information: Other information applicable to both method development and establishment and/or to routine sample analysis could include:

- Lists of abbreviations and any additional codes used, including sample condition codes, integration codes, and reporting codes.
- Reference lists and legible copies of any references.
- SOPs or protocols covering the following areas:
- Calibration standard acceptance or rejection criteria
- Calibration curve acceptance or rejection criteria
- Quality control sample and assay run acceptance or rejection criteria
- Acceptance criteria for reported values when all unknown samples are assayed in duplicate
- Sample code designations, including clinical or preclinical sample codes and bioassay sample code
- Assignment of clinical or preclinical samples to assay batches
- Sample collection, processing, and storage
- Repeat analyses of samples
- Reintegration of samples

Protocol Guidance: The following provides guidelines/tools that should be used to define method performance[31]:

General Protocol: Prepare and analyze method blanks, matrix blanks, reference materials (if available) and matrix spikes (using matrix blanks if available) of known concentration as generally described .Accuracy or bias and precision are calculated from these results. Data will also be used to evaluate matrix effects and ruggedness/robustness of the method resulting from changes in the sample matrix.

The following general validation tools should be used to generate method performance characteristics as described in the Performance Characteristics section below.

Blanks: Use of various types of blanks enables assessment of how much of the result is attributable to the analyte in relation to other sources. Analyze blanks and compare these results to the limit of detection.

Reference materials and certified reference materials: The use of known reference materials (when available and applicable) can be incorporated to assess the accuracy or bias of the method, as well as for obtaining information on interferences[32].

Matrix Blank: A substance that closely matches the samples being analyzed with regard to matrix components. Matrix blanks are used to establish background level (presence or absence) of analyte(s) and to verify that sample matrix and equipment used does not interfere with or affect analytical signal. 1.

Matrix Spikes (Laboratory Fortified Matrix): Recovery 2. determinations can be estimated from fortification or 3. spiking with a known amount of analyte and calculation of 4. spike recoveries. (Note: spike recovery may not be truly 5. representative of recovery from naturally incurred 6. analytes.) Matrix effects can also be assessed with these 7. samples. Accuracy or bias and precision are calculated 8. from these results. The data can also be used to evaluate 9. robustness of the method resulting from changes in the 10. sample matrix. 11.

Incurred Samples: Samples that contain (not laboratory 12. fortified) the analyte(s) of interest (if available) may also 13. be used to evaluate precision and bias (if analyte concentration(s) are reliably known). Analyte recovery can also be evaluated through successive extractions of the sample and/or comparison to another analytical procedure with known bias.

STANDARD OPERATING PROCEDURE

Standard Operating Procedures (SOPs) are to be available for all routinely used sampling or analytical laboratory methods. The Laboratory must maintain a log of all SOPs in use and must maintain a file of all revisions of SOPs used in the past. A current list of all SOPs and revision number and date must be appended to the Laboratory QA Plan. All such methods shall be documented in detail. Generally, simply citing a published method is not adequate for a SOP.

Published methods rarely have all the procedural details, and those that do generally have to be modified for the applications or facilities at hand. Suggested references for the format of SOPs are included in the reference section of this document (6, 8). These SOPs shall be prepared in document control format[34].

As a minimum the following items should be included:

* Title Page
* Scope and Applications
* Definitions
* Procedures
* QC Limits
* Corrective action Procedures, Including Procedures for Secondary Review
* Documentation Description and Example Forms
* Miscellaneous Notes and Precautions
* References

At times certain SOPs may not cover all the above elements, especially

administrative type SOPs. In that case some other format and elements should be developed to properly address the purpose of documenting the procedure covered by the SOP. SOPs shall be located in an accessible place(s) and copies shall be available to all personnel needing them to perform their duties. Once prepared, these SOPS would only have to be included in QA project plans by reference, after being subjected to prior review and approval. This section will be quite flexible and will include all the lab's SOPs for all its' routine operating and analytical procedures. The following is a list of possible SOPs and should not be considered to be all inclusive[35]:

Evidentiary SOPs
Sample Receipt and Storage
Sample Preparation
Glassware Cleaning
Calibration (Balances)
Analytical Procedures (for each analytical System)
Maintenance Activities (for each Analytical System)
Analytical Standards
Data Reduction Procedures
Documentation Policy/Procedures
Data Validation/ Self Inspection Procedures
Data Management and Handling
Quality Assurance and Quality Control
GC/MS Operating Conditions

Preconcentrator: The following are typical cryogenic and adsorbent preconcentrator analytical conditions which, however, depend on the specific combination of solid sorbent and must be selected carefully by the operator[36].

Sample Collection Conditions
Cryogenic Trap Adsorbent Trap
Set point - 150EC Set point 27EC
Sample volume - up to 100 mL Sample volume - up to 1,000 mL
Carrier gas purge flow - none Carrier gas purge flow - selectable

Desorption Conditions: Cryogenic Trap Adsorbent Trap
Desorb Temperature - 120EC
Desorb Flow Rate - 3 mL/min He
Desorb Time - <60 sec

The adsorbent trap conditions depend on the specific solid adsorbents chosen.

Trap Reconditioning Conditions: Cryogenic Trap Adsorbent Trap
Initial bakeout 120EC (24 hrs) Initial bakeout Variable (24 hrs) .After each run 120EC (5 min), After each run Variable (5 min).

GC/MS System: Optimize GC conditions for compound separation and sensitivity. Baseline separation of benzene and carbon tetrachloride on a 100% methyl polysiloxane stationary phase is an indication of acceptable chromatographic performance.

The following are the recommended gas chromatographic analytical conditions when using a 50-meter by 0.3-mm I.D., 1 µm film thickness fused silica column with refocusing on the column.

Item Condition
Carrier Gas : Helium
Flow Rate : Generally 1-3 mL/min manufacturer
Temperature Program : Initial Temperature: -50EC
Initial Hold Time : 2 min

Ramp Rate : 8E C/min
Final Temperature : 200EC
Final Hold Time : Until all target compounds elute.

SUMMARY

The efficient development and validation of analytical methods are critical elements in the development of pharmaceuticals. Success in these areas can be attributed to several important factors, which in turn will contribute to regulatory compliance. Experience is one of these factors-both the experience level of the individual scientists and the collective experience level of the development and validation department. A strong monitoring and training program is another important factor for ensuring successful methods development and validation. Companies must maintain an appropriate level of expertise in this important dimension of developing safe and effective drugs.

Gas chromatography-mass spectrometry is the single most important tool for the identification and quantitation of volatile and semivolatile organic compounds in complex mixtures. GC-MS is widely used for the quantitation of pollutants in drinking and wastewater and also used for the quantitation of drugs and their metabolites in blood and urine. For the residual solvent detection GC-MS is used when compared to LC-MS. This is important for samples that have been in the field, held in storage for months, and for highly weathered samples and the most typical use of GC-MS in the petrochemical industry it is for process troubleshooting.

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