Appendix 3: Statistical Analysis

Analytical results observed in the laboratory are to be accurate and reliable based upon statistical principles and by the current Good Manufacturing Practices. ORA LAB Manual Vol. III Section 4, *Basic Statistics and Data Presentation,* covers statistical analysis in detail and should be reviewed. The following information is supplemental to LAB Manual, Vol. III. sec. 4 and is provided as a summary and a guide.

A. Definitions

Some of the definitions are from the ICH Validation Procedures definitions, which appeared in the Federal Register #60, March 1, 1995, p. 11260.

Number of Significant Places: The total number of digits, not counting leading zeros, from the first non-zero digit, to the last digit known with a reasonable confidence.

Error: Difference between the expected and the encountered value of some variable.

Incertitude: Lack of availability of information about a reading caused by the impossibility of reading closer than a certain readout limit. For example, a digital readout of 22.57, with no digits beyond the second decimal, has incertitude of +/- 0.01.

Standard Deviation, $\sigma = \sqrt{[\Sigma(x_i - x_{average})^2/(n-1)}$. See *Precision*.

RSD: Relative standard deviation. The standard deviation divided by the average; usually expressed as percent by multiplying it by 100.

Accuracy: The closeness of agreement between the value which is accepted (either as a conventional true value or as an accepted reference value) and the value found.

Precision: The closeness of agreement between a series of measurements obtained from multiple sampling under the same conditions. Usually expressed as standard deviation.

Repeatability: Precision measured in the same laboratory and under the same operating conditions, over a small time period.

Intermediate precision: Precision within the same laboratory but under different conditions, analysts and equipment.

Reproducibility: Precision between different laboratories, such as in collaborative studies.

Robustness: The ability of a procedure to remain unaffected by small but deliberate variations in method parameters. It is an indication of reliability during normal usage.

B. Using Significant Digits (see ORA LAB Manual, Vol. III, Sec. 4.3.2 Significant Figures)

Normally, the confidence in the value of a number decreases from the first to the last digit. Thus if a volume measurement gives 10.567 mL we may be quite confident that the value is close to 10 mL but less confident about the decimal "5", still less about the "6" and not really sure at all about the "7". In this case, the digit 7 is called the *least significant digit* (LSD). The number 10.567 is said to have 5 significant digits. The number of significant digits is obtained counting all digits that give reasonable confidence, not including leading zeros. For example, 0.0031 has two significant digits.

The rules of significant digits are rules of *data communication.* Only report the digits in which there is confidence. The non-significant ones are eliminated according to the rules outlined below. Data in process are kept with an extra significant figure and rounded to the correct significant figures for the final answer. A good rule is using five digits throughout, but reporting at the end with the correct number of significant digits.

Note: Do not confuse significant digits, with *significant places*. The latter depend on the units used and the concept should be avoided in this context. For example 12.53 cm = 125.3 mm. Both have four significant digits, but different number of significant places (two and one respectively).

C. Performing Rounding (see ORA LAB Manual, Vol. III, Sec. 4.3.1 Rounding of Reported Data)

The procedure (see USP) for rejecting the digits beyond the least significant digit, LSD, is the following:

1. Establish the position of the LSD.

2. If the digit to the right of the LSD is less than 5, eliminate all remaining digits after the LSD.

3. If the next digit is larger than or equal to 5, increase the LSD by one and eliminate all the digits beyond the LSD.

Remember: The calculations are always retaining an extra significant figure over the significant ones. Rounding should be done only at the end to the correct significant figures.

D. Using Average and Standard Deviation (see ORA LAB Manual, Vol. III, Sec. 4.2.2 Error and Deviation; Mean and Standard Deviation)

The average and standard deviation are estimates *after the fact*. This means that a number of measurements are first made and then their statistical properties are obtained from the analysis of the set of measurements. Calculations:

Average = $\Sigma(x_i)/n$, Sum of all data divided by the number of data

Variance = $\Sigma(x_i-x_{average})^2/(n-1)$ The number n-1 is the number of data minus one.

Standard deviation, $\sigma = \sqrt{[\Sigma(x_i-x_{average})^2/(n-1)]}$

%RSD = Relative Standard Deviation = (standard deviation)/(average)*100

3.7 Answer Key

3.3 Methodology

1. **What are the five most significant items of information in the USP "General Notices?" Compare the "General Notices" with the introductory chapter in the Official Methods.** This is a subjective answer but the answer could include the following: The use of "Official" and "Official Article"; Significant Figures and Tolerances; Reference Standards/Reagents; Tests and Assays; Preservation, Packaging, Storage and Labeling (especially storage temperature); Weights and Measures; and Concentrations. The "Guide to General Chapters" contains information regarding procedures used throughout the USP; the General Notices serves as a general guidance document.

2. **Using the current USP, find the Dissolution procedure for Ascorbic Acid Tablets and how does it differ from most other Dissolution procedures?** Ascorbic Acid Tablets monograph uses a pooled sample procedure where equal volumes of each vessel are pooled and the one-pooled solution is assayed. The assay procedure is performed on the solution from each vessel for most Dissolution procedures.

3. **Where in the USP are the listings for column types used in HPLC and GLC? How are they designated?** There is a separate section "Chromatographic Columns". This lists both the column used for each general chapter or monograph and also the column types for

both HPLC and GLC. HPLC is listed as *packings* with a letter designation beginning with 'L'. GLC is listed as *phases* with a letter designation beginning with 'G'.

4. **What does the term "Official Compendium" mean? Name the three major types of "official" methods used by FDA.** "The term 'official compendium' means the official United States Pharmacopeia, official Homeopathic Pharmacopeia of the United States, official National Formulary, or any supplement to any of them." See Section 3.5 References (7) Chapter II Definitions Sec. 201 (j), three major types of "official" methods used by FDA are the (1) United States Pharmacopeia, (2) AOAC Official Methods. (3) FDA approved New Drug Application or Abbreviated New Drug Application methods.

3.4.1 Pharmaceutical Products Overview

1. **When would you use a USP standard and when would you use a working standard? What is needed for working standards that is not needed for USP standards and why?** The USP standard when availableis to be used for the analysis of all USP products. Working standards are used for products and dosage forms not found in the USP or when an USP standard is not available. The purity and identity of all working standards are to be verified before use to assure that the standard is sufficiently pure and it is the correct material. The standard purity is to be used in the assay calculations. The USP standard is assumed to be 100% unless otherwise stated on the label.

2. **What is the procedure in the laboratory for obtaining USP standards? NIST standards? Controlled drug standards?** The answer to this question is dependent on the District and Laboratory policy. The trainee is to describe this procedure and reference the Laboratory's SOP.

3. **Describe the differences between an immediate release tablet/capsule, an extended release tablet/capsule and a delayed release tablet. How would one classify transdermal patches and implants?** Immediate release tablet and capsules release the active ingredient within a small period of time, typically less than 30-minutes. Extended Release tablets release the active ingredient at a sustained and controlled release rate over a period of time. Typically extended release tablets and capsules release their ingredient with time periods of 8 hours, 12 hours, 16 hours, and 24 hours. Delayed Release tablets release the pharmaceutical dosage after a set time. These are frequently enteric coated to prevent release in the stomach thus release the dosage in the intestinal track. Transdermal patches and implants would be classified as extended release products, but release the dosage over a much greater period of time such as weeks or months.

4. **What items would likely be found in compressed tablets and what is their purpose? In capsules? Name at least three items.**
    - Active Ingredient – supplies pharmacological dose: any drug product.

- Diluents (Fillers) – provide bulk: lactose, calcium phosphate, microcrystalline cellulose.
- Binders – impart cohesive properties: starch, povidone (PVP).
- Lubicants – reduce mechanical friction and prevent tablets from sticking to punches and dies: magnesium stearate.
- Glidants (Flow Agents) – promotes free flowing characteristics: colloidal silicon dioxide, talc.
- Disintegrants – enable tablets to break apart in aqueous environments: starch, crospovidone (PVP-XL), croscarmellose, Na starch glycolate.
- Capsule – holder for active and filler materials: gelatin.

5. **Describe where you would find the PAC code for a product** All FDA collected samples appear in FACTS, which lists the PAC codes and the type of sample. PACs for the various programs are located in the Compliance Program Guidance Manual (CPGM). The CPGM frequently provides additional sample information including methodology to be used for the products being analyzed.

3.4.2.1 Basic Analytical Techniques

1. **What other standard technique can be used for determining the Melting Range and why are these used? What standards are used for calibration of the Apparatus?** A USP Reference Standard or National Formulary Reference Standard corresponding to an authentic specimen of the substance being tested is added in equal parts to the substance until an intimate mixture is obtained. Agreement of observations on the authentic and the mixture provides reliable evidence of chemical identity. USP Melting Point Reference Standards are used for calibration of the apparatus. There are six such standards: Acetanilide, Caffeine, Phenacetin, Sulfanilamide, Sulfapyridine and Vanillin. The one that melts nearest the expected melting temperature of the substance under test is used.

2. **Why are limit and qualitative tests added to USP monographs? Compare the limit tests of the Acetaminophen product with those found in the Dextrose (including those not run). Which tests use the same method?** USP monographs serve to define the identity, strength, quality, and purity of the substances for which they are written. Limit and qualitative tests are part of that determination. Tests for the presence of foreign substances and impurities are provided to limit such substances to amounts that are unobjectionable. Both monographs use the same tests for residue on ignition <281>, chloride <221>, and sulfate <221>. Acetaminophen also has limit tests for water (<921>Method I), sulfide, heavy metals (<231>Method II), readily carbonizable substances <271>, free p-aminophenol, and p-chloroacetanilide. Dextrose has limit tests for water (<921>Method III), Arsenic (<211>Method I), heavy metals (231), dextrin and soluble starch sulfites.

3. **Both the Acetaminophen product and Dextrose have a method for Water. What is the difference between the two methods? Can the Acetaminophen method be used for Dextrose? Can the Dextrose method be used for Acetaminophen? Explain the answers.** The method used for water determination in Acetaminophen is titrimetric. There is a quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer, which reacts with hydrogen ions (Karl Fischer Reagent – sulfur dioxide & iodine dissolved in pyridine & methanol). A gravimetric method is used for water determination in Dextrose. The Dextrose is dried at 105 degrees for 16 hours. The hydrous form loses between 7.5% and 9.5% of its weight. The anhydrous form loses not more than 0.5% of its weight. The method for Acetaminophen and Dextrose are not interchangeable because one has to account for the water of hydration.

4. **Look at the USP general section on heavy metals <231>. What is the analyte of interest? Explain why Method I is used for Dextrose while Method II is used for Acetaminophen.** The test is used to determine the content of metallic impurities that are colored by sulfide ion, specifically lead (percentage by weight). The substance being tested is visually compared to a control Standard Lead Solution. The sample is calculated as total Heavy Metals equivalent to lead. Method I is used for substances that yield clear/colorless preparations under the specified test conditions. Method II is used when the resulting preparation is not clear/colorless or for substances that interfere with the precipitation of metals by sulfide ion or for fixed and volatile oils. In Method II, sample is initially introduced into a crucible and charred.

3.4.2.2(I) Ultraviolet/Visible Spectrophotometry

1. **Define: absorbance, absorptivity, molar absorptivity. (See USP<851> for more information.)**

   Beers Law supplies the terms: $\log_{10}(1/T) = A = abc$

   - Absorbance: [A] is the logarithm, to the base 10, of the reciprocal of the transmittance (T).
   - Absorptivity : [a] – the quotient of the absorbance (A) divided by the product of the concentration of the substance (c) expressed in grams per liter, and the absorption path length (b) in cm.
   - Molar Absorptivity: [Symbol like a rounded capital E] -- the quotient of the absorbance (A) divided by the product of the concentration, expressed in moles per liter, of the substance and the absorption path length in cm. It is also the product of the absorptivity (a) and the molecular weight of the substance.

2. **What are the typical cell size, specimen concentration and absorbance range used in the analysis of a substance in the UV or visible range?**

- Typical cell size (path) is 1 cm.
- For many pharmaceutical substances ‘concentrations of about 10 ug of the specimen per ml often will produce absorbances of 0.2 to 0.8 in the ultraviolet or the visible region.’ Ref. USP <851>.

3. **What do the expressions "similar preparation" and "similar solution" indicate (as used in tests and assays involving spectrophotometry in the USP)?** The expressions "similar preparation" and "similar solution” indicate that the reference specimen, generally a USP Reference Standard, is to be prepared and observed in a manner identical for all practical purposes to that of the test specimen. Ref. USP <851>.

4. **What do the expressions "concomitantly determine" and "concomitantly measured," indicate (as used in tests and assays involving spectrophotometry in the USP)?** The expressions "concomitantly determine" and "concomitantly measured" indicate that the absorbances of both the solution containing the test specimen and the solution containing the reference specimen, relative to the corresponding test blank, are to be measured in immediate succession. Ref. *USP <851>*.

5. **Good practice demands that comparisons be made at the wavelength at which peak absorption occurs. What difference in nm for the wavelength specified in the USP monograph is considered acceptable?** If the difference is more than +/- 1 nm from the specified wavelength, re-calibration of the instrument may be indicated. Ref. USP <851>.

3.4.2.2(II) Fourier Transform Infrared Spectrophotometry (FTIR)

1. **What is FTIR?** A computerized process in which the source of energy is sent through an interferometer (instead of a monochromator) and onto a sample which is irradiated. An interferogram (intensity vs. time spectrum) is obtained, which has the characteristics of a spectrum. A computer mathematically converts the (intensity vs. time) spectrum (fourier transform) into an (intensity vs. frequency) fourier transform infrared spectrum.

2. **What is the advantage of an FTIR spectrometer (interferometer) over a conventional (dispersive) spectrometer (with a prism or grating monochromator)?**

Conventional/Dispersive	Interferometer
• A large number of moving parts • Requires 7 1/2 minutes to 30-minutes for one scan • Slow scan speed • To improve resolution adjust slits • No internal reference for frequency accuracy • Stray light within the instrument • Thermal problems because sample is close to IR source • IR radiation by sample is viewed by the detector	• Only the mirror is in motion • Produces a spectrum in as little as 1 second • Rapid scan speed • No slits in the system to define resolution • Laser provides an internal calibration system with frequency accuracy • No equivalent to stray light • Sample further removed from the IR source • Emitted radiation by the sample is not viewed by the detector

3. **What is the purpose of the interferometer in the FTIR spectrometer?** The interferometer replaces the conventional monochromator so that energy throughput is inherently greater (no slits) and all wavelengths present reach the detector during the entire time needed to observe a spectrum. The interferometer is coupled to a detector and a computer that reduces the data to an ordinary spectral curve faster.

4. **List the types of sample preparation techniques used for analysis with an FTIR spectrometer and conventional/dispersive spectrometer, and explain when they would be used.**
    - Mulls formed by grinding the compound and dispersing it in mineral oil (nujol). The oil/sample mixture is then placed between salt plates and pressed together.
        - Poor solubility in a useful solvent and saving of time in sampling handling.
    - Gases and vapors in gas sample cells produce spectra, which differ fundamentally from those observed in a condensed state (solids, liquids, solutions).
        - Sample is to be in a gaseous state that can be transferred to a gas sample cell.
    - Solutions by dissolving the compound (liquid or solid) in a solvent and placing it in a sealed liquid cell.
        - Sealed cell is perhaps the simplest technique to use for liquids with viscosities less than or similar to water.
    - KBr discs (pellets) prepared using a press.
        - A dried powdered or crystalline sample is ground with KBr.
    - Attenuated Total Reflectance (ATR)
        - A solid sample place *in situ* on diamond/crystal center.

3.4.2.2(IV) Optical Rotation/Polarimeter

1. **Describe polarimetry and the types of products for which it is used.** Polarimetry is the measurement of optical rotation. Many pharmaceutical substances are optically active in the sense that they rotate an incident plane of polarized light so that the transmitted light emerges at a measurable angle to the plane of the incident light. Ref. USP<781>. Pharmaceutical products that show a dextrorotatory, or (+) optical isomers, and levorotatory, or (-) optical isomer. The products that show such optical rotatory power are chiral.

2. **What is the purpose of the solid phase calibration cell and how is it used?** The solid phase calibration cell is used for checking the calibration of the polarimeter. This calibrator consists of a plate of quartz mounted in a holder perpendicular to the light path. The calibrator standard readings are traceable to NIST. The cell replaces the need to prepare Dextrose and Sucrose solutions that also can be used for calibration. The solid phase calibration cell is stabilized to a temperature of 25^{o}C and placed in the instruments cell holder. The degree of rotation of the cell can be read directly on the instruments readout system.

3. **What is the general equation used in polarimetry and how does temperature effect polarimeter readings?** The general equation for polarimetry is $[\alpha]^{t\lambda}$ where 'α' is specific rotation at 't' temperature λ wavelength, which equals 100a/lc where 'a' is the observer rotation in degrees, 'l' is the path length in decimeters and 'c' is the concentration in grams per 100 ml. Temperature is in the equation and has a direct effect on the measured specific rotation. Temperatures need to be maintained within 0.5^{o}C of the stated value in the USP for the calculations to be valid for a measured product.

3.4.2.3(I) Column Chromatography

1. **When and why would someone use column chromatography?** Column chromatography offers a wide choice of stationary phases and is useful for the separation of individual compounds, in quantity, from mixtures." Ref. USP <621>. This technique is used for color analysis and when large quantities of purified materials are needed.

2. (**To be done at the completion of each chromatography sections.). Prepare a chart, comparing and relating each of techniques used in chromatography for the following: column materials, separation theory, phases, equipment needed, ease of use, accuracy, sampling and sampling techniques, automation, accuracy, and quantitation.** *See Q & A Attachment A*

3.4.2.3(II) Thin Layer Chromatography (TLC)

1. **TLC is a qualitative method. How can someone use this technique as a semi-quantitative tool? As a quantitative tool?** A visual comparison of the size of the spots may serve for semi-quantitative estimation. Quantitative measurements are possible by means of densitometry, fluorescence, and fluorescence quenching; or the spots may be carefully removed from the plate, followed by elution with an effective solvent and spectrophotometric measurement. Ref. USP <621>.

2. **The exercise uses UV light as a visualizing tool. What other visualizing tools are commonly used for TLC?** The spots produced by paper or thin-layer chromatography may be located by: (1) direct inspection if the compounds are visible under white or either short-wavelength (254 nm) or long-wavelength (360 nm) UV light; (2) inspection in white or UV light after treatment with reagents that will make the spots visible. Ref. USP <621>.

3. **In exercise 2 above, what was the smallest spot seen? In the larger spots, were other 'breakdown' or 'related substances' spots seen in the chromatogram? If found, can the quantity be estimated based on the size of the smaller standard spots?** (Result from experiment reported here.) Most of the USP monographs that use the TLC technique for chromatographic purity call for preparing a reference solution with a known concentration, usually a low concentration. For example, if a standard of about 2ug is prepared and a breakdown spot is observed, an analyst can estimate the quantity as "NMT 2 ug" if the R_f and the intensity of the secondary spot are close to the values of the reference standard solution.

4. **(To be done at completion of each chromatography section.) Prepare a chart, comparing and relating each of techniques used in chromatography for the following: column materials, separation theory, phases, equipment needed, ease of use, accuracy, sampling and sampling techniques, automation, accuracy, and quantitation.** *See Q & A Attachment A*

3.4.2.3(III) Gas Chromatography (GC)

1. **What type of products can be tested by GC? What products cannot be tested? Describe a GC method that can analyze products that normally cannot be tested by a GC method.** GC is a chromatographic technique that can be used on any pharmaceutical product that contains volatile organic compounds of interest. Typical tests may include assay, impurity testing and organic volatile impurity analysis. The product is to be volatile above the lower temperature limit of the stationary phase and below the upper limit of the stationary phase. Products which are not volatile organic compounds cannot be tested without further treatment. For non-volatile compounds (for example sugars) and thermally labile compounds (for example antibiotics) derivatization can be used to transform normally non-volatile or thermally unstable compounds to a volatile compound usable for GC.

2. **Describe how a Flame Ionization Detector works. Describe at least three other GC detectors commonly used. Which detector is similar to FID and how is it used?**
    - A FID detector consists of an air/hydrogen flame and a collector electrode. Ions collected produce an electrical signal.
    - Electron-Capture detector (contains a radioactive source of ionized radiation) is very sensitive for compounds containing halogens and nitro groups.
    - Thermal Conductivity detector (employs a heated wire placed in the carrier gas stream) is used for all volatile compounds but has very low sensitivity. This is sometimes considered to be the universal detector since it can detect most volatile compounds regardless of structure.
    - Nitogen-Phosphorus or alkali flame-ionization detectors (contains a thermionic source such as alkali-metal salt or rubidium glass bead) are very sensitive for organic nitrogen or phosphorus compounds but have low sensitivity for other hydrocarbons.
    - Mass Spectrometer detector is used to identify ion fragments of the parent molecule. This is a very sensitive detector that can give molecular structural information for the compound of interest.
    - The Nitrogen Phosphorus Detector is a variant of the FID. If the same sample and column are used with an FID and NPD, the same chromatogram will be produced except the NPD will ignore the hydrocarbon solvent and other non-nitrogen containing components and produce a much simpler chromatogram. Used for nitrogen containing drugs.

3. **Describe capillary GC and how it differs from packed column GC. What are the advantages and disadvantages of each technique?**
    - Capillary GC columns are long (25 - 50 meters), thin (0.18 to .53mm ID) fused silica columns with higher separation efficiency (more theoretical plates) and superior resolution than that of a packed GC column (typically 2 meters long and ¼ inch diameter).
    - Advantages are their high efficiency and sturdiness. They are easy to handle and do not break as easily as glass columns. The capillary column is widely used for complex mixtures because of their superior resolution
    - The disadvantages of capillary GC is that the columns can become overloaded, which affects the chromatography and it needs a modified inlet or injection port. Capillary columns are also generally more expensive.

4. **When is it appropriate to use temperature programming? What are the advantages of using temperature programming?** If there is a wide range of boiling points, the analyst needs to increase the column temperature over time in order to elute the high boiling point compounds of interest. Besides decreasing the time needed for analysis, temperature programming provides improved peak shapes, and because later peaks are now taller and sharper, it provides better detection.

5. **GC commonly uses four different gases. Air, Nitrogen, Helium and Hydrogen. What is the purpose of each gas, how is it used and at what flow rates?**
    - Air is typically used as the oxidant to support the flame in a FID detector. Typical flows are 350 – 450 mls/min.
    - Nitrogen and Helium are typically used as carrier gases. The type of carrier gas used is dependent on the detector used. For capillary and megabore columns, typical flows are 0.5 – 10mls/min, for packed columns 8 – 40 mls/min. Nitrogen and helium can also be used as make-up gas in an FID detector when using capillary columns. Make-up gas + column flow in a FID detector typically should be 25- 30mls/min.
    - Hydrogen is typically used as the fuel to support the flame in a FID detector. Typical flow rates are 30 ml/min. It is also sometimes used as a carrier gas due of its high efficiency in temperature programming as per the van Deemter curve.

6. **(To be done at completion of each chromatography section.) Prepare a chart, comparing and relating each of techniques used in chromatography for the following: column materials, separation theory, phases, equipment needed, ease of use, accuracy, sampling and sampling techniques, automation, accuracy, and quantitation.** *See Q & A Attachment A*

3.4.2.3(IV) High Performance Liquid Chromatography (HPLC)

1. **Describe a typical 'basic' HPLC system and the purpose of each component.**
   A typical liquid chromatograph consists of:
    - Reservoir - to contain the mobile phase.
    - Solvent Delivery or Pumping System – causes the mobile phase to flow through the system at a specified flow rate. Single or multi-pump designs are can be purchased.
    - Injector - to introduce the sample into the mobile phase. Autosamplers are commonly used.
    - Column - component in which the separation is achieved by different mechanisms such as partition, adsorption, or ion-exchange of compounds in the test solution between the mobile and stationary phases.
    - Detector – detects or measures compounds as they elute from a column. Common detectors include Single Wavelength UV, Diode Array/UV, Refractive Index and Fluorescence.
    - Data collection device - to receive and store detector output – usually a computer or integrator.

2. **What is the difference between normal phase and reverse phase? List at least three column types for each phase.**
    - Normal phase: polar stationary phases and nonpolar mobile phase
    - Reverse phase: non-polar stationary phase and polar mobile phase.
    - Examples of column type for each phase:

Reverse Phase: C18, C12, C8, C4, C2, C1, Phenyl
Normal Phase: Silica, CN, NH_2, PAC, Diol, Alumina

3. **For the chromatograms obtained in exercise 2 'Acetaminophen and Caffeine Tablet' calculate the following for each of the peaks: Retention Time, Retention Volume, Relative Retention Time to IS, Capacity Factor, Resolution, Tailing, Theoretical Plates, Height Equivalent Theoretical Plate, Peak Widths at base, half height, and 5% height. Also calculate one set of sample results using both peak area and peak height calculations. Are the results different? If so explain why differences may result.**
   (Formulas and calculations to be shown) Example given below: (Student is to perform exercise using information obtained from experiment.)

HPLC Exercise

Note: Electronic integrators perform most of the following calculations in automated systems. However, it is important to verify if the formula used by the system conforms to the USP

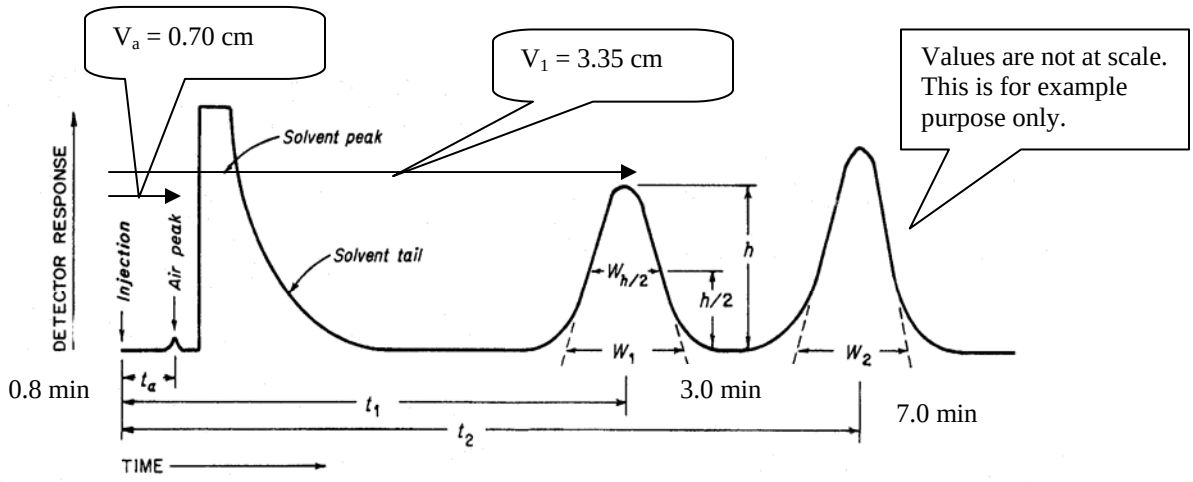

Figure 1: Chromatographic separation of two substances

In which V_a and V_1 are the retention volumes for the non-retained component and the compound under test, respectively. And t_1 and t_2 are the retention times measured from time of injection to time of elution of peak maximum.

Capacity Factor Calculation (*k'*)

$$k' = \frac{t_1}{t_a} - 1 = \frac{3.0\,\text{min}}{0.8\,\text{min}} - 1 = 3.8$$ USP <621>

Express k' in terms of the amount of mobile phase it takes to elute each compound off the column using the retention volumes.

$$k' = \frac{V_1 - Va}{Va} = \frac{3.35cm - 0.70cm}{0.70cm} = 3.8$$

Relative Retention Time (R_R) of Peak 2 with respect to Peak 1

$$R_R = \frac{t_2}{t_1} = \frac{7.0\,\text{min}}{3.0\,\text{min}} = 2.3$$ USP <621>

Resolution between Peak 1 and Peak 2

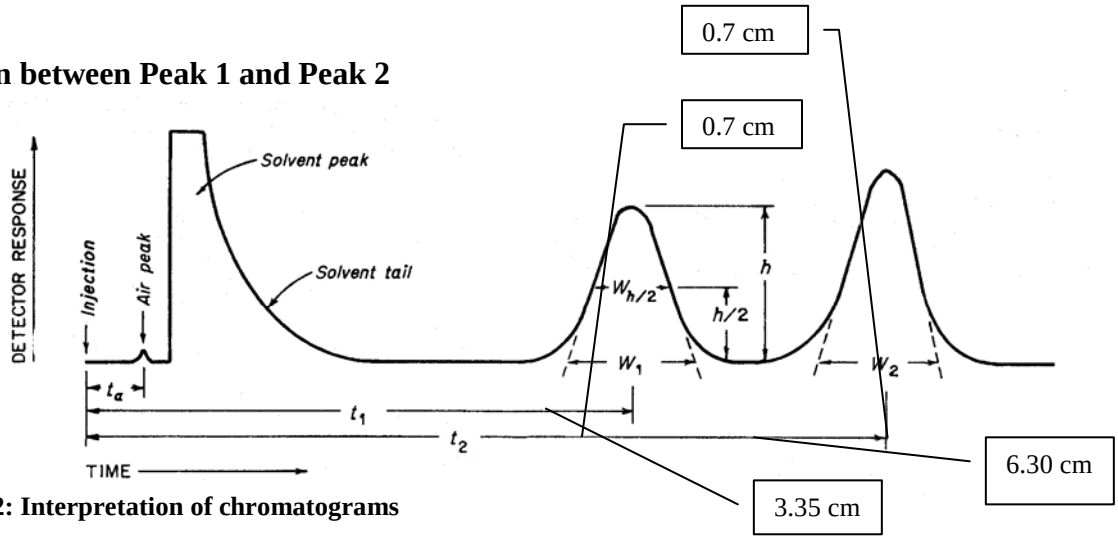

Figure 2: Interpretation of chromatograms

$R = \frac{2(t_2 - t_1)}{W_2 + W_1}$ (The retention times and the widths have the same units) USP <621>

$R = \frac{2(6.30cm - 3.35cm)}{0.7 + 0.7} = 4.2$

Note: The USP provides another formula to determine resolution and the number of theoretical plates that are convenient when electronic integrators are used. Refer to USP <621>

Tailing Factor (T) Calculation USP <621>

$$T = \frac{W_{5\%}}{2f} = \frac{12.0cm}{5.0cm \times 2} = 1.2$$

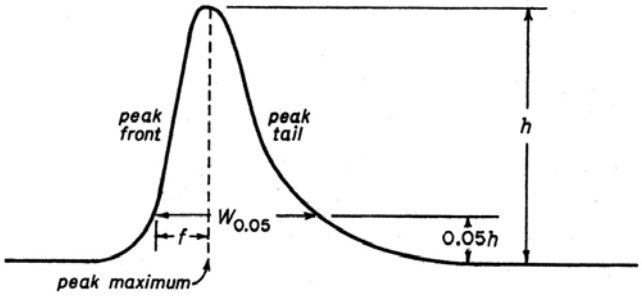

Figure 3: Asymmetrical chromatographic peak

Number of Theoretical Plates (N) USP <621>

Values from Figure 2

$$N = 16\left(\frac{t}{W}\right)^2 = 16\left(\frac{3.35cm}{0.7cm}\right)^2 = 366.4$$

Note: The USP provides another formula to be used with the value of $W_{h/2}$, the peak width at half-height. There is also in the USP another convenient formula to be used when electronic integrators are used.

Calculations using peak areas and peak heights USP <621>

"Peak areas and peak heights are usually proportional to the quantity of compound eluting. These are commonly measured by electronic integrators but may be determined by more classical approaches. Peak areas are generally used but may be less accurate if peak interference occurs." Ref. " USP <621>.

4. What is the purpose of the internal standard used in exercise 2? Calculate one set of sample results without using the internal standard. Are the results different? If so explain why differences may result.

A major source of error in quantitative comparison is irreproducibility in the amount of sample injected, notably when manual injections are made with a syringe, in the case of GC methods. In these cases, the effects of variability can be minimized by addition of an internal standard. Today's automatic injectors greatly improve the reproducibility of sample injections and reduce the need for internal standards. Ref. USP <621>.

Following, a theoretical calculation example using an internal standard:

Injection	Area of Analyte	Area Internal Standard	Response Ratio (Analyte/Internal STD)
STD	38.14009	35.60928	1.07107
Inj1	37.02482	36.19063	1.02305
Inj2	29.65308	27.93705	1.06142
STD	38.72269	34.96597	1.10744

Average of STD Bracket: 1.07274

Concentration of the STD (C): 10.143 mg in 50 mL volumetric flask: 0.20286 mg/mL
Label Claim of the product: 5 mg
Dilution Factor: 100
Sample weight: 0.36135 mg
Average tablet weight (ATW): 0.09059 mg

To determine the quantity, in mg, of the portions of tablet taken:

$$mg = 100C\left(\frac{R_u}{R_S}\right)\left(\frac{\text{ATW}}{\text{Sample Weight}}\right)$$

Where R_u and R_s are the ratios of the peak responses of the corresponding analyte and internal standard peaks obtained from the Assay preparation and the Standard preparation, respectively.

Example for injection 1

$$mg = 100 \times 0.20286\left(\frac{1.02305}{1.07274}\right)\left(\frac{0.09059}{0.36135}\right) = 4.85mg$$

Percent of label claim: 4.85/5 = 97.0%

Example for injection 2

$$mg = 100 \times 0.20286\left(\frac{1.06142}{1.07274}\right)\left(\frac{0.09059}{0.36135}\right) = 5.03mg$$

Percent of label claim: 5.03/5 = 100.6%

Without using the internal standard…

We used peak responses instead of the ratios:

$$mg = 100 \times 0.20286\left(\frac{37.02482}{38.43139}\right)\left(\frac{0.09059}{0.36135}\right) = 4.90mg$$

Percent of label claim: 4.90/5 = 98.0%

$$mg = 100 \times 0.20286\left(\frac{29.65308}{38.43139}\right)\left(\frac{0.09059}{0.36135}\right) = 3.92mg$$

Percent of label claim: 3.92/5 = 78.4%

THERE IS A DIFFERENCE!
Sample and standard solutions that do NOT contain an internal standard CANNOT compensate for variations in injection systems, injection volumes and sample/standard solutions.

5. **When and why would someone use gradient elution in HPLC? What is the effect of temperature on HPLC? Would temperature programming such as found in GC be effective for HPLC?** Ref. USP <621>.
   - "The technique of continuously changing the solvent composition during the chromatographic run is called gradient elution or solvent programming. It is sometimes used to chromatograph complex mixtures of components differing greatly in their capacity factors. By increasing the mobile phase strength over time during the chromatographic separation the separation time between components is decreased."
   - "Retention time in all LC separations generally decreases with increase in temperature. Another effect is the increase of the column efficiency. At elevated temperatures, viscosity of liquids decreases and the diffusion coefficient increases. However, *temperature effects in HPLC are not as significant as in gas chromatography*. Volatile solvents cannot be allowed to rise to higher temperatures, and the high temperature may influence the stability of the attached bonded ligands on the adsorbent surface. The common temperature range for HPLC is from ambient temperature up to 60 or 70 degrees C.
   - Temperature programming is not effective for HPLC.

6. **(To be done at completion of each chromatography section.) Prepare a chart, comparing and relating each of techniques used in chromatography for the following: column materials, separation theory, phases, equipment needed, ease of use, accuracy, sampling and sampling techniques, automation, accuracy, and quantitation.** *See Q & A Attachment A*

3.4.2.4(I) Dissolution

1. **What are the parameters that need be checked and corrected before dissolution can be run? Describe what effect each would have on an analysis.**
    - Temperature is to be at 37° C +/- 0.5°C (unless otherwise specified).
        - Temperatures higher or lower can effect the rate the product goes into solution
    - Rotation speed of paddle or basket.
        - The faster the speed the faster the product is likely to go into solution.
    - Paddle or basket height or positioning.
        - Both are specified in the USP as 25 +/- 2mm from bottom of paddle/basket to interior vessel bottom. The distance can have a great affect for paddles which relates to the distance from the product. The affect is minimal for the basket where the product is within the basket.
    - Other Physical Parameters (level, wobble or runout, centering, vibration, verticalaity or perpendicularity).
        - If these parameters are not correct, they can alter the dissolution rates generally resulting in erroneous results.
    - The vessel also needs to be checked physically for proper dimensions, cleanliness, irregularities and for micro-fractures or scratches in the glass.
        - Improper dimensions and irregularities can cause turbulence and interferences.
    - Each method in the USP is developed using a specified set of parameters. The 'Q' value and general limits of each (extended release) product are established using the parameters listed. If any parameter is not correct then the Dissolution analysis is not valid.

2. **Exercise 2 demonstrates a profile analysis. When would this technique be used? For what type of products could this technique be used? Look in the USP and list a monograph that uses a profile type analysis.** Use this technique to determine the full time range in which a tablet or capsule dissolves. This is frequently used to develop dissolution methods and determine the optimal time that the 'Q' value is established. A modified form of the profile technique is used for controlled release products, where a sample solution is drawn at set times over an extended period. Frequently used for 6, 8, 12, 16 and 24 hr extended release capsules and tablets. Also Transdermal Delivery Systems use modified procedures that determine a profile of a product over a period of time. The USP <724> describes several methods and acceptance tables for Extended-Release, Delayed Release and Transdermal Delivery Systems. Products include Clorpheniramine Maleate Extended Release Capsules, Aspirin Extended Release Tablets, and Nicotine Transdermal Systems.

3. **Describe the reason and procedure for removing air from the dissolution media?** Dissolved gases can cause bubbles to form, which may change the results of the test. Ref. USP <711>. One method of de-aeration is as follows: Heat the medium, while stirring gently

to about 41°C. Immediately filter under vacuum using a filter having a porosity of 0.45 um or less. Provide vigorous and continuous stirring of the filtrate under vacuum for 5 minutes. Media must be de-aerated immediately before use. Gently transfer the medium directly to the vessel. Do not introduce air into the medium. Other validated de-aeration techniques for removal of dissolved gases may be used. Ref. USP <711>. There are also commercial apparatus that use a vacuum to draw the medium through a fine pinhole into a large holding vessel.

4. **Why not use water for all dissolution media and have constant paddle/basket rotation speed for all determinations?** Many drugs are insoluble or have limited solubility in water. Buffers dilute acids and surfactants are routinely used for Dissolution medium. They increase dissolution rates and stabilize the pH of resulting solutions. Test conditions such as pH and surface tension can vary depending on the source of water and may change during the dissolution test itself, due to the influence of the active and inactive ingredients. The paddle/basket speeds can affect the rate of dissolution, generally the faster the speed the faster the dissolution. Methods have been developed to optimize the paddle/basket speeds so that a satisfactory 'Q' value is obtained within a specified time.

5. **Can one compare in-vitro dissolution results with in-vivo clinical studies? Explain the answer.** Yes. In general an in-vitro, in-vivo (IVIV) correlation has been recognized. It is the goal of the pharmaceutical scientist to find a relationship between an in-vitro characteristic of a dosage form and its in-vivo performance. An effort to connect dissolution and pharmacokinetics is often referred to as "in vitro-in vivo correlation" (IVIVC) analysis. Numerous IVIVC studies can be found in the literature. Controlled release products, rather than immediate release products, are the focused in the IVIVC literature. Since only products with dissolution rate-limited absorption (and with complete complete absorption) can be expected to exhibit a slope of one and zero intercept (Y=mX) mX). The relationship is not as well defined for immediate release products, but depending on the in-vitro release rate and the in-vivo adsorption rate, the two methods are generally recognized as satisfactory for comparison.

3.8 Document Change History

Version 1.3	Revision	Approved: 11-03-05	Author: LMEB	Approver: LMEB
Version 1.4	Revision	Approved: 01-17-08	Author: LMEB	Approver: LMEB
Version 1.5	Revision	Approved: 02-02-10	Author: LMEB	Approver: LMEB
Version 1.6	Revision	Approved: 02-06-12	Author: LMEB	Approver: LMEB
Version 1.7	Revision	Approved: 02-13-14	Author: LMEB	Approver: LMEB

Version 1.3 changes:
Table of Contents – Added 3.6 Appendix 2; added 3.8
Revised Section 3.1, 3.2 B., 3.3 B. 5., 3.4.1, 3.4.2.1 A., B. & C., 3.4.2.2, 3.4.2.2 I. B., 3.4.2.2 II. B., 3.4.2.2 III., 3.4.2.3 III. B. & IV. B., 3.4.2.4 I. A. & II., 3.4.3, 3.5, 3.6 Appendix 1 & 3; added 3.6 Appendix 2.

Version 1.4 changes:
3.1, 3rd para, last sentence – Deleted “altogether”
3.2, 3rd para - Changed “diethyl” to “diethylene”
3.3 A. 1st para – added “or pressure” after high performance
3.3 B. 1., 4th para – revised
3.3 B. 6. – revised
3.3 B. 8. – updated 5th web address
3.3 C. 2. – revised
3.4.2.3 B. 3. – deleted
3.4.2.3 III. C. 2. – added (FID)
3.4.2.4 I. C. 3. – revised 5th sentence
3.5 – deleted 1st para
3.7 – revised 3.3.2, 3.4.2.2. (I) 1. & 5., 3.4.2.2 (II) 4., 3.4.2.3. (III) 1. & 4., 3.4.2.3. (IV) 2., 3.4.2.4 (I).1.

Version 1.5 changes:
3.3.B. – updated web links in 5., 7., & 8.; added last sentence to 8.
3.4.2.4 I. A. III. B. – revised
3.4.2.4 I. B. 1. – revised
3.4.2.4 I. B. 2. - deleted
3.5 – updated web links
Footer – updated web link

Version 1.6 changes:
3.3 B. 2. – changed “testing” to “review” in second sentence
3.3 C. 5. – deleted
3.4 – added last sentence
3.4.2 I.. A. – added USP reference to bullet 7; deleted bullet 10; added bullets 13 and 14
3.4.2.I. B. 2. – deleted Disintegration Test from bullet 2
3.4.2.I. B. 3. – “6” changed to “8” in bullets
3.4.2.2 II. A. III. – added D. and E.
3.4.2.3.III B. 1. – changed “Suspension” to “Solution” in 1.; added Method 11 b to bullet
3.4.2.3 III B. 2. – revised
3.4.2.3 IV. A. 2. – added 7.; added “Gradient” to C.4.
3.4.2.3.IV. B.1. – “Suspensions” changed to “Solution”; B change to A, part 5.1 in second bullet; 5.1 deleted in third bullet
3.4.2.4. I. C – deleted 2.
3.4.2.4.II. 16. – changed VIII to VII
Appendix 1 I. – deleted 2.3; changed Apparatus 2 to Apparatus 1 in 3.2; changed Suspension to Solution in 5. ; changed 6.1 B. to HPLC; deleted 6.6, 6.7, and 6.8; 7.1 changed to FTIR; 7.6 spelling corrected and changed to FID; deleted 7.10; added “loss on” to 8.5; added <281> to 8.6; 8.12 changed to color from limit test; 9.2 changed from UV to HPLC; added 10.

Appendix 2 I. – added 4. and changed GC product and test method
3.7 Answer Key 3.3 3.- answer revised and deleted 4., 5., and 6.
3.7 Answer Key 3.4.1 1. – first and second sentence revised
3.7 Answer Key 3.4.2.1 – 3. deleted sentences 3., 4., 5., and the last sentence
3.7 Answer Key 3.4.2.4.(I) –deleted 2.

Version 1.7 changes:
Header – Division of Field Science changed to Office of Regulatory Science
7. – Divison of Field Science changed to Office of Regulatory Science