*ANALYTICAL METHODS FOR PHARMACEUTICAL ANALYSIS A TRAINING MANUAL**

by

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SECTION I
PREPARATION OF SAMPLES AND STANDARDS

SAMPLE AND STANDARD PREPARATION FOR PHARMACEUTICAL DRUG FORMULATIONS

In your career as a drug analyst, you will handle many types of pharmaceutical formulations. Space does not permit describing how to handle all differenttypes of formulations. However, a few of the most common types will be described, along with a discussion of standard preparation. It is important to remember that no matter what type of formulation is encountered, the primary goal of the analyst is to obtain a representative and homogeneous sample for analysis.

TABLET SAMPLES

Analysis of tablets is usually straightforward but the analyst must be aware of rogue samples that are not amenable to the method outlined. Proceed as follows:

a. At least 20 tablets are weighed accurately on a four-place balance. The weight is recorded and the average tablet weight is calculated.

b. The tablets are ground with a mortar and pestle, sieved through a 40-60 mesh drug sieve, and mixed throughly. All grinding should be done in a hood to prevent inhaling any drug.

c. A portion is weighed into a volumetric flask, solvent is added, and the mixture is shaken (heat or ultrasound may be used if needed) to extractthe active drug substance from the tablet matrix. The solution is allowed to adjust to room temperature, and diluted to volume (quantitatively, if needed). The solution should be filtered before further dilutions are made. With HPLC methods, in which membrane filters are used, it may be easier to filter the solutions if they are diluted first. It may be necessary to prefilter solutions if they won't go through a 0.45 m membrane filter. If the solution is prefiltered, there is a possibility that some active ingredient may be absorbed by the filter.

NOTE--If a large quantity of solid material is weighed and remains insoluble in the solvent, it is possible that less than the volume declared on the volumetric flask is present because some of th volume has been occupied by the solid. In this case the sample should be weighed into a container and an accurate volume of solvent should be added.

Some tablet samples may have to be handled slightly differently.

Sugar-coated, enteric-coated, or time-delay tablets may pose hidden problems to the analyst. For example, colored sugar coatings, which interfere with the analysis, may have to be washed off and the tablets dried before weighing and grinding; exceptionally hard tablets may require use of a WIG-L-BUG (trade name, Crescent Inst. Co.), which uses steel balls, to reduce them to a fine powder; if grinding generates enough heat to make the tablet matrix sticky or causes the active ingredient to decompose, the composite may be prepared by putting 10 or 20 tablets into solution directly to prepare the material for analysis.

CAPSULE SAMPLES

There are two types of capsules: hard gelatin and soft gelatin. Hard gelatin capsules are made up of two parts that slip together to hold a dry powder or small beads; they are called dry powder capsules (DPC) or timed-release capsules (TRC). Soft gelatin capsules are one piece and usually contain a liquid or syrupy substance such as a vitamin preparation.

Dry powder capsules. Twenty capsules are accurately weighed and the contents of each are emptied into a container. The shells are cleaned by swabbing them either with a Q-tip or a solvent (e.g., ethyl ether) that dissolves the powder but does not attack the shells. The powder is mixed and saved for analysis, and the dry empty shells are weighed. The average weight of the dry powder is obtained by subtracting the average weight of the shell from the average weight of the entire capsule. The dry powder is then handled in a manner similar to the ground tablet material.

Timed release capsules. These are handled in a manner similar to the dry capsules except that the shell generally does not need to be cleaned. However, the beaded material must be weighed and then reduced to a homogeneous powder, by grinding, sieving, and mixing before analysis.

Soft gelatin capsules. Usually these are carefully sliced open with a scalpel and the liquid material is collected in a volumetric flask, with care not to spill any of the material. The inside of the capsule is then rinsed

with a suitable solvent contained in a syringe with a fine gauge needle. The rinsings are collected in the volumetric flask. The soft gelatin capsule is then discarded. Usually several capsule contents are combined as a composite and the result is calculated on the basis of an individual capsule.

LIQUID SAMPLES

Liquid samples may be either solutions or suspensions. Both types of samples should be mixed before sampling, but in the case of suspensions which quickly separate out, such as some suspensions for injection, it is extremely important to mix the sample and then immediately remove the portion for analysis with a pipette. In addition, viscosity of liquid pharmaceutical formulations can vary from non-viscous (water-like) to very viscous (suspensions or magmas). Some of these formulations are solutions for injection, elixirs, syrups, etc. Samples of low viscosity are measured by using a TD (To Deliver) pipette and diluting to volume with an appropriate solvent. With more viscous samples, a TC (To Contain) or a Mohr wide-bore calibrated pipette may have to be used; then, after draining, the remaining material is rinsed out of the pipette with a suitable solvent. If samples are too viscous to be pipetted, it may be necessary to weigh them and convert to volume, using a specific gravity determination.

OTHER TYPES OF SAMPLES

There are many other types of pharmaceutical formulations including creams, lotions, ointments, dental pastes, inhalators, dermal patches, implants, etc. Each of these can pose different and sometimes difficult problems to the analyst. For example, a cream or ointment should be removed from its container before sampling, rapidly and thoroughly mixed with a spatula on a glass plate, and then returned either to its original container or to another container (in the case of samples that come in tubes).This guide is not intended to be all-encompassing but to make the analyst aware of normal procedures used for sampling. It is imperative that the analyst obtain a homogeneous and representative portion of sample for analysis. Many times erratic or erroneous results can be traced to faulty preparation of the sample for analysis.

STANDARDS

Drying. If a USP method is used, the monograph specifies the drying method. (NOTE--In the case of USP Reference Standards, the bottle label contains the method to be used for drying). However, sometimes the methods are changed, and these changes are noted in the USP Supplements. If there is a difference in instructions, those in the monograph should be followed.

Since many drug standards are costly or in short supply, only the quantity needed for the analysis should be dried. Some standards require elevated temperatures; others are dried at room temperature over silica gel. The drying time may be specified or the instructions may specify drying to constant weight. All dried standards should be kept in a desiccator until the analysis has been completed.

Weighing. Whenever a secondary standard (previously analyzed bulk sample) is available, it should be used. The secondary standard should be evaluated before use. See section 7 of the GLP manual for more information on handling standards. Caution: A check analysis requires the use of the USP standard. Standards come in concentrated form (in most cases, 100% of active ingredient) and are usually fine powders or crystalline materials. After drying, such samples will be subject to static electricity and may have a tendency to jump around when handled with a spatula tip. The static electricity may be discharged with anti-static guns, which are available at some of the balance tables.

Caution: A DUST MASK SHOULD BE WORN TO PREVENT BREATHING ANY DUST!

When possible, quantities of 25 mg or more should be weighed on a four-place analytical balance. Quantities less than 25 mg (e.g., 10 mg or less), should be weighed on a semimicro or micro balance. Weighing by difference is the technique most often used. Quantitative transfer is also acceptable.

Dissolving. The weighed sample is placed in a volumetric flask and the specified solvent is added until the flask is approximately half-filled. The flask may be put on a shaking machine or may be sonicated until the sample has dissolved. The flask and contents are allowed to come to room temperature and diluted to the correct volume. If further dilutions are required, they must be done quantitatively. For HPLC analysis, dissolving and diluting with the mobile phase is preferable. However, with drugs that must be dissolved in another solvent to prevent degradation or hydrolysis, all dilutions are made with the recommended solvent. It should be kept in mind that the dissolving solvent must be compatible with the mobile phase and not cause any buffering agents to precipitate out during the HPLC run. Also, with drugs that are slightly soluble in the mobile phase, care must be taken not to inject too large an amount of the drug so that it precipitates out in the

column. The solution to be assayed is filtered through a membrane filter of no greater than 0.5 mm porosity. Concentrations of standard and sample active ingredient should be kept nearly the same so that areas or absorbances will be comparable.

SECTION II
ANALYTICAL WEIGHING

ANALYTICAL WEIGHING

All chemists have weighed materials in the laboratory and recognized the importance of the operation. The primary goal in all weighing is to be accurate because unless it is done correctly, the analytical results will be meaningless. This training module is aimed at pointing out some basic operations which will improve the accuracy of the measurement. Modern balances are electronic with digital readout. These balances produce fast and reliable results when properly handled and cared for. Good Laboratory Practices are the rule in all weighings.

ACCURACY IN WEIGHING

The validity of any analysis depends on an accurately weighed sample. Three types of balances are currently used for drug analysis, namely, micro, semimicro, and analytical. The semimicro and analytical balances can be combined into a single instrument by changing the balance sensitivity. The analytical balance weighs gram quantities to four places, the semimicro balance weighs to five places, and the micro balance weighs to six places. The amount of sample required and the balance capacity needed determine the type of balance used. Since all electronic balances read the weight directly, the analyst is likely to become complacent and assume that all readings are correct at all times. NOT SO!

Electronic balances depend upon a force to compensate the mass; this force depends in turn on location and environment. The balance must be placed on a vibration-free table located in a temperature-stable room free from drafts and corrosive vapors. Balances of the electronic type must be calibrated. Laboratory balances are usually calibrated by a balance service technician but these services may be performed at wide intervals. A calibration check should be done at frequent intervals. The laboratory should have a set of calibrated weights so that the analyst can easily check the calibration. THE CHECK SHOULD BE MADE WITH CALIBRATED WEIGHTS. A calibrated 100 mg weight can serve as a very useful check on the balance and give confidence in the balance readout.

The area around the balance should always be kept clean. Spills should be avoided, but if a spill occurs, it should be cleaned up immediately. All weighing vessels, handling tools, flasks, bottles, or anything else used around the balance must be clean and dry.

Weighings are done by difference to minimize errors. The weight of the tare is subtracted from the combined weight of the tare plus the sample. Electronic balances are equipped with a zero tare feature which allows setting the tare at zero so that only the weight of the sample is displayed.Several precautions can improve your accuracy, as follows:

1. Turn on the balance and allow it to warm up. If the balance has been turned off for a time, allow it to stabilize at least 60 minutes before making any measurement. Better still, leave the balance turned on permanently.

2. Brush any dust and dirt from the balance pan before and after any weighing. Check that the weighing chamber is clean.

3. Select a container as small as possible to hold the sample. Since the balance to be used depends upon the sample size and the balance capacity, do not exceed the capacity. Make sure that all containers and handling tools are clean and dry.

4. Before making any weighing, check the zero point and adjust accordingly. If this is not done, a zero point error will be included in the sample weight.

5. Keep the balance chamber closed at all times except when adding or removing the objects to be weighed, as changes in temperature, humidity, or air currents will alter the reading.

6. Handle all tare containers or objects to be weighed with either tweezers or tongs. Handling with the fingers can change the temperature or leave a grease smudge that will alter the weight. If it is necessary to handle the tare, use finger cots or gloves.

7. Make readings without delay. Allow only enough time for the sample to come to equilibrium and the display to stabilize.

8. Do not weigh any hot or cold objects, as the result will not be correct. Make sure that the temperatures of the weighed objects are at the temperature of the balance.

9. Many samples are finely divided powders which have been dried; they may be subject to static electricity which causes the particles to jump or fly around. Before weighing, discharge the static electricity with an antistatic gun. Be careful not to inhale the powders. Wear a suitable mask while handling materials of this nature. Static will be a problem when the humidity goes below 30% RH.

10. All samples will be affected by surface moisture; the amount will depend on the humidity of the laboratory. Weigh the sample at ambient temperature.

11. Volatile liquids are subject to evaporation. Weigh volatile materials in closed containers with the cap tightly sealed. Weigh hygroscopic materials in sealed, capped bottles to prevent water take-up.

12. Remove all items used around the balance area and clean up any debris. Always leave the balance area CLEAN! Keep the balance door closed at all times except when adding or removing sample.

SECTION III

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1. HPLC training
2. Preparation of mobile phase for HPLC
3. Quantitative HPLC analysis
4. Column care
5. Troubleshooting in HPLC analysis

HPLC TRAINING MODULE

High-performance liquid chromatography is well-suited to the analysis of drugs and drug preparations because it can provide fast, precise, and accurate results for a wide variety of organic materials. HPLC is used for the analysis of a large fraction of the drug samples handled by FDA, and it is likely that your work in the laboratory will require this analytical technique. This training aid is intended to help you quickly to acquire the skills that you will need to carry out analyses by HPLC.

TRAINING COURSES

The FDA has available training materials which can help you to learn or review the essentials of quantitative HPLC analysis. Even if your previous education and experience include some background in this technique, you may find it profitable to spend some time with written, audio, and visual instructional materials. Below is summarized the contents of tape-slide courses which are available for this purpose. You can tailor your instruction to your needs by viewing those courses or portions thereof which are necessary. Each course module takes about one hour to view. Spend as much time as necessary with each course, until you thoroughly understand the principles of the technique.

SAVANT Tape-Slide Course 1, "Principles of High Performance Liquid Chromatography"

- Introduction or review of chromatography, especially liquid chromatography
- (LC) Definition of chromatography and special terms
- Advantages and limitations of HPLC
- Basic instrumentation
- Major column types
- Judging the quality of chromatograms

SAVANT Tape-Slide Course 4, "Qualitative and Quantitative HPLC"

- Qualitative HPLC
  - Identification of components by retention time, "spiking"
  - Identification by spectroscopy (UV, MS, IR, NMR, etc.) of eluents
- Quantitative HPLC
  - Peak heights
  - Peak areas
  - "Spiking"

- Precision and accuracy
- Sampling
- Sample preparation
- Chromatography
- Integration-- manual and automated
- Calculation

Calculation Methods

- Simple normalization (assumes same detector efficiency)
- Corrected area normalization
    - External standards
    - Internal standards
    - Standard additions

(SAVANT Tape-Slide Course 2, "HPLC Instrumentation", Course 3, "Column Selection in High Pressure Liquid Chromatography", and Course 5, "Troubleshooting in Liquid Chromatography" are not recommended for beginning analysts.)

PREAMBLE TO LABORATORY PRACTICES

Even more than most methods of analytical chemistry, HPLC analysis requires thoughtful pre-planning if laboratory time is to be used efficiently to produce precise and accurate results. Careful planning of the analysis will also reward the analyst by eliminating wasted effort and frustration. Some important elements in HPLC planning are:

1. If the sample or the standard to be analyzed requires treatment (such as drying) before the analysis is begun, this procedure can be started and continued while other preparations for the analysis are underway.

Analyses of some samples require several hours drying, so plan accordingly.

2. If the procedure to be used requires changing the mobile phase, or if the column to be used was stored with a solvent incompatible (immiscible) with the mobile phase you intend to use, it will be necessary to purge the column and the detector by flushing them with an intermediate solvent, miscible in both the new and old mobile phases, then the new mobile phase. If you are changing mobile phases, the whole system will need to be flushed and filled with the new mobile phase. If you are unsure about the compatibility of solvents, a simple test of their miscibility in a test tube is advised.

3. It is important that the mobile phase be filtered and degassed before it is introduced into the apparatus. These operations are normally accomplished simultaneously by filtration through a glass-frit funnel having a porosity of 0.45 mm coupled to a vacuum pump and filter flask.

4. It is wise to prepare enough of the mobile phase so that, even if some unanticipated problems arise, it is unlikely that it will be necessary to prepare an additional batch of mixed, filtered, and degassed solvents. For most analyses, one to two liters will be sufficient.Most drug analyses require the use of buffer or salt solutions in the mobile phase. RINSE OUT THE COMPLETE SYSTEM WITH WATER FOR AT LEAST 1 HOUR WHEN SHUTTING DOWN THE OPERATION FOR OVERNIGHT. For longer storage, the instrument and the Waters Intelligent Sample Processor (WISP) must be cleaned with water and water/alcohol mixture. Make sure that the water wash is done first because the organic solvent may precipitate any salt.

PRACTICE ANALYSIS

Before beginning the laboratory exercise below, you should have developed the volumetric and gravimetric skills typically required in the preparation of a sample and of mobile phases for HPLC. If you are not confident that your skills in these techniques are adequate, you should stop here and review or learn afresh those procedures, because one cannot expect to obtain accurate results from an instrumental method unless the quantitative techniques used for sample preparation are of commensurate quality.

Many FDA samples are analyzed by methods in the United States Pharmacopoeia (USP). A USP method which is representative of HPLC analysis is the assay of aspirin tablets (USP XXII, page 113). The purpose of the procedure is to determine the amount of active ingredient in aspirin tablets and to ascertain the concentration of free salicylic acid, a decomposition(hydrolysis) product. You should perform the analysis under the supervision of an experienced chemist. This analysis is known as a reverse-phase separation under isocratic flow since the mobile phase is polar, the stationary phase is nonpolar and the flow is constant. The separation type is BPL or bonded phase liquid chromatograpy. This type of separation is made on the basis of differences in the polarity of the compounds. The more polar compounds elute first.

All of your work must be recorded on a standard worksheet, just as if this analysis were not just a practice exercise. See the Training Module on Worksheets.

GETTING STARTED

PRELIMINARIES TO GETTING STARTED

Every two months, a list of current USP reference standards is published in Pharmacopoeial Forum. Consult the most recent list to determine the appropriate standards for aspirin and salicylic acid. Dry both standards over silica gel for five hours. Dry only the quantity needed as follows: Roughly weigh out the quantity of standard needed for analysis from the USP Reference Standard bottle into a closable container. Return the USP Reference Standard bottle to stock. The time of drying is a minimum so additional drying will not harm the drugs. Both standards could be dried overnight in a desiccator over silica

gel to expedite the analysis. Keep the STANDARDS in a desiccator until the analysis has been completed.

PREPARATION OF MOBILE PHASE AND DILUTING SOLVENT

Start with making up the mobile phase and the Diluting Solvent.

MOBILE PHASE

Prepare the mobile phase for this analysis by dissolving 2 g of sodium 1-heptanesulfonate in 850 mL of water and 150 mL of acetonitrile. (NOTE--the volumes can be measured in graduate cylinders and the volumes are measured separately and not additively). After mixing thoroughly, adjust the pH to 3.4 with glacial acetic acid. Adjust the pH as follows: Pour the water solution into beaker and stir with a magnetic stirring bar. Follow the pH with a reliable pH meter. NOTE--Always calibrate the pH meter before making any measurement on the mobile phase. Calibration is made by using standard pH buffers especially in the pH range that you are interested in. Adjust the meter to read the pH of the standard buffer, then the meter is ready for the measurement of the mobile phase. Carefully and slowly add the acid by means of a disposable pipette equipped with a small rubber bulb. Do not add too much acid. Vacuum filter the solution through a 0.45 mm membrane filter on a sintered-glass support to remove any dirt and to degas. The filter system consists of a vacuum flask and a sintered-glass filter funnel. The vacuum is applied by means of a pump. Prepare sufficient quantity of mobile phase to complete the analysis; however, if you shut down the instrument overnight and still have mobile phase left for additional runs, you must sonicate the mobile phase to remove any dissolved air. Your final run will require at least 800 mL to complete the analysis.

DILUTING SOLVENT

Prepare 1 L of "Diluting Solvent", consisting of a mixture of acetonitrile and formic acid in the ratio 99:1.

INSTRUMENT SETUP

It is necessary to set up the instrument conditions before

actually preparing the samples for analysis because some drugs may be unstable if allowed to stand around for any extended period (aspirin fits this behavior). The chromatographic analysis is performed using an HPLC instrument which includes a column, pump, injector, recorder, and a UV detector set at a wavelength of 280 nm. The column is 4.0 mm x 30 cm, packed with packing L-1 (a C18, octadecyl silane chemically bonded to porous silica or ceramic particles 5 to 10 micrometers in diameter). Column dimensions should be: internal diameter, from 3.9 to 4.0 mm, and length, from 25 to 30 cm.Turn on the power to the WISP, Data Module, and UV detector and allow 20 to 30 minutes for the instruments to warm up. Attach a filter to one end of a piece of supply tube, place the filter in the mobile phase, and attach the other end of the tube to the inlet supply of the pump. Prime the pump with the mobile phase and pump at 2 mL per min. to clear the system of any residual solvents. Continue the pumping until you have established uniform flow by observing the output to be steady and without pulsing. The flow and pulsing can be observed by directing the flow into a beaker. A check on the pump output can be measured at this time by collecting the liquid in a graduate cylinder in a fixed time. Shut off the pump and connect the column to the output of the WISP and to the input of the detector with the flow in the direction as marked on the column.

NOTE --The specific instrument settings described here apply only to instruments using a Waters Data Module and WISP. With different equipment you would set the parameters to achieve the same HPLC conditions.

The column must be conditioned and equilibrium established before making any solutions. Set the pressure cutoff on the pump to a value below the maximum pressure recommended for your column. Set the flow at 0.2 mL per min; turn the pump on again and gradually increase the flow until you reach 2 mL per minute. Watch the pressure on the column and make sure that the pressure does not exceed that recommended by the supplier. Set the response of the UV detector to 0.1 aufs (absorbance units full scale) and monitor the output on the Data Module. The flow and the response are monitored on the Data Module with the parameter 03 set at 1 for continuous recording (1= continuous recording and 0 = recording only during HPLC run). Continue the flow until you get a good base line -- one with zero slope and no noise. Watch the pressure on the column and observe if the pressure remains constant. Begin now to establish conditions for the analyses. Allow the instrument to continue running while you prepare the solutions, but you may want to cut back the flow to conserve the mobile phase, for example, 0.5 mL per minute.

INSTRUMENT CONDITIONS

Prepare a solution for making preliminary measurements to establish instrument conditions, reproducibility and suitability of the chromatographic system.These conditions can be established with a solution of a "Secondary Standard" of aspirin and salicylic acid prepared as described under STANDARD SALICYLIC ACID PREPARATION. Filter this solution as follows: Select a 0.45-mm membrane filter that won't dissolve in your solvent (see NOTE below). Place the filter in a Swinnex adapter. Draw about 5 mL of the solution into the syringe. Attach the filter adapter to the syringe. Push about 1 mL of solution through the syringe and discard the filtrate. Discharge the remaining solution through the filter into a WISP vial.

NOTE--HPLC for drug analyses mostly requires use of mixed organic and aqueous solvents, but sometimes pure organic solvents are used. To be sure that the solvent and filter are compatible, use the following guide:

Filter type	Compatibility
Nylon	Used with most organic or aqueous solvents
Teflon	Used with organic solvents only.

The nylon filters are recommended since drug analyses use mostly organic /aqueous solvent mixtures. Do not use filters made of cellulose esters since they might dissolve.

Use the default settings of the WISP and Data Module with the exception of RUN TIME which is set at 20 minutes for the first scouting run and set for a single injection. Reset the flow on the pump gradually to the original 2 mL per min. Allow time for the system to give a noiseless, drift-free baseline. Start the run by pressing the RUN switch. Observe the chromatogram and make adjustments to obtain a suitable chromatogram. You will need to change chart speed, RUN TIME, sensitivity of the detector, and volume of injection. The volume of injection and the sensitivities of the detector determine the height of the peak. Higher injection volume will raise the peak height but raising the setting on the UV detector, i.e., going from 0.1 auf to 0.2 auf, will lower the response. Reset the injection volume and/or the UV sensitivity to alter the peak height so that it is suitably displayed on the chart. Then set the run time on the WISP corresponding to 2 minutes greater than the time when the chromatogram returns to the base line. Set in any new parameters

on the Data Module using the instructions found in the Data Module Manual. Make another injection and observe the chromatogram. Continue making additional injections to optimize the curves and integration. The Data Module Manual gives a clear description of the steps necessary to achieve proper integration (you may alter the integration by changing parameters 22 (noise rejection) and 23 (area rejection) on the Data Module).

Optimization of the separation and integration conditions should produce a chromatogram which should be similar to the figure in Addendum D. Slight differences may exist between your curves and the one illustrated because of differences in all parameters. You will observe peaks due to difference between the solvents in the mobile phase and the solvents in your injected solutions, and due to the compounds; you will observe narrow or up and down tick marks due to integration. A blank space is observed in the middle of the down side of the peak when proper integration is achieved. The integration will not be correct if the blank space appears anywhere else on the chromatogram. After establishing the optimum conditions, chromatograph at least six injections of the preliminary solution. Discard the first run and use the next 5 runs for the calculations. The procedure for calculations is found in Addendum C. Make calculations for tailing factor, resolution, relative standard deviation, relative retention time, and plate count. The acceptable values for each of these are listed with the respective equations.

ANALYSIS OF SAMPLES

The optimum parameters have been established and now you are ready to begin analysis of samples. The samples will be run under the same conditions, so keep everything running while you prepare the samples.

ANALYSIS FOR ASPIRIN AND SALICYLIC ACID

USP XXII, page 113 describes the analysis of aspirin by HPLC. You should be familiar with this reference. Many drug samples hydrolyze after solutions are prepared, which could cause the analytical results to change as a function of time. This is true for the aspirin which partially hydrolyzes to salicylic acid. It requires considerable time to complete all runs because of the many injections; therefore, careful planning is required to minimize hydrolysis. READ OVER THE INSTRUCTIONS AND CHECK ADDENDUM B TO SEE THE ORDER OF STEPS TO FOLLOW. STANDARD ASPIRIN PREPARATION The "Standard Aspirin Preparation"is made by

dissolving an accurately weighed quantity of USP Aspirin Reference Standard in sufficient Diluting solution to obtain a solution having a known concentration of approximately 0.5 mg/mL. Accurately weigh 125 mg of USP Aspirin Reference Standard and dissolve in 250 mL of Diluting Solution. You will need a portion of this solution for the salicylic acid analysis as well as the assay.

STANDARD SALICYLIC ACID PREPARATION

Obtain the current USP Salicylic Acid Reference Standard. Accurately Weigh approximately 15 mg and dissolve in 100.0 mL of the Aspirin Standard Preparation. Pipet 5.0 mL of this solution into a 50-mL volumetric flask, dilute to volume with the Standard Aspirin Preparation, and mix. This dilution method is used so that a standard four-place analytical balance can be used and a high degree of accuracy maintained. You will now have a standard solution of salicylic acid with a concentration of approximately 0.015 mg/mL.

SAMPLE PREPARATION

Run the sample in duplicate by making two separate weighings and solutions.Accurately weigh 20 tablets, record the total weight and calculate the average tablet weight (ATW, in mg). Grind these 20 tablets to a fine powder and sieve the powder through a 60-mesh screen. (Regrind any particles retained on the sieve until all material passes through). Thoroughly mix the powder. Transfer an accurately weighed quantity equivalent to 100 mg of aspirin to a suitable container. (NOTE--100 mg of sample will not be equivalent to 100 mg of aspirin because the final dosage drug will have some excipients present). Add 20.0 mL of Diluting Solution and sonicate for 15 minutes. Centrifuge to settle any undissolved material. This solution will be termed the "Aspirin Sock Solution". Proceed immediately to the salicylic acid determination. After completing the salicylic acid determination, continue with the aspirin analysis on page 10.

SALICYLIC ACID DETERMINATION

Separately chromatograph the Stock Solution of Aspirin and the Standard Salicylic Acid Solution using the conditions and parameters established in the preliminary setup. Filter all solutions, using the Swinnex adapter, into the vials of the WISP. Make three separate injections of Standard Salicylic Acid Solution, then two separate injections of the Aspirin Stock Solution, and finally two separate injections of the Standard Salicylic Acid Solution. The first injection data are discarded.

Average the results from each pair of injections. Calculation of the percentage of salicylic acid in the aspirin tablets is accomplished as follows:

(Cs) X (5 mL/50 mL) X (20 mL /Wt Spl, mg) X (ATW/DW) X (Au/As) X 100 = % salicyclic acid in the aspirin tablet,
where Cs = concentration of Standard Salicylic Acid, mg/100 mL
ATW = average tablet weight, mg
DW = declared weight of drug per unit
Au = area under the HPLC peak for the sample
As = area under the HPLC peak for the standard

The maximum allowable percentage of salicylic acid for uncoated tablets is 0.3%.

When you inject the Aspirin Stock Solution, you will get a peak for salicylic acid and the peak for the aspirin. The aspirin peak will be very intense and may be off the chart. (The data module will integrate even though the peak is off chart). The salicylic acid peak will be very small compared to the aspirin peak. (NOTE--It is possible that no salicylic acid peak will be seen in the assay sample because of no detectable quantity). The response for the aspirin is ignored in the calculation for the salicylic acid. You will also notice an extra peak soon after injection; this peak is due to the difference between the solvent in the injected solution and the solvent in the mobile phase.

ASPIRIN ASSAY

Quantitatively dilute an accurately measured volume of the Aspirin Stock Solution as follows: Pipette 10.0 mL of the Aspirin Stock Solution into a 100-mL volumetric flask. Add Diluting Solution to correct volume and mix. The final solution is known as the "Assay preparation".

Make three separate injections of the Standard Aspirin Preparation and the Assay Preparation; disregard the data from the first injection. Make two separate injections of each of the Assay Preparations. Record the chromatograms and measure the responses expressed as areas for the major peak. Average the areas obtained from each pair of injections. Calculate the

percentage of aspirin ($C_9H_8O_4$) found in the tablet compared to the declared amount.

(Cs) X (20 mL/Wt Spl, mg) X (100 mL/10 mL) X (ATW/DW) X (Au/As) X 100 = % of drug in aspirin tablet

where Cs = concentration of the Standard Aspirin, mg/100 mL
ATW = average tablet weight
DW = declared weight of drug in aspirin tablet
Au = area of sample under HPLC peak
As = area of standard under HPLC peak.

For Aspirin Tablets, the allowable percentage of the labeled amount per tablet is 95 to 105%

Other Training Modules

Other modules that will aid the chromatographer are:

1. Care and Storage of Columns
2. Sample Preparation
3. Good Laboratory Practices and Safety
4. Record Keeping
5. Troubleshooting in HPLC
6. Quantitative HPLC
7. Mobile Phase Preparation

ADDENDUM A

Injection of solutions

All samples must be filtered to remove dust or other particles before injection to remove anything which might plug or damage the columns. The filtering is done as previously described for the trial run. Solutions are injected onto the column by one of two methods depending upon the instrumentation. The methods are:

(1) Manual injection
(2) Automatic injection

Manual Injection

Load the solution by means of a syringe having a needle of the correct size for the injector assembly. Draw excess solution into the syringe, remove bubbles and set syringe to the desired injection volume. Switch the valve indicator to the "LOAD" position. Insert the loaded syringe into an injector valve assembly having a fixed-volume loop. Push the syringe to force the solution into the injector loop. Make sure that the loop is loaded by observing an outflow of solution. Switch the valve manually to the "INJECT" position to make the injection. The valve remains in the "INJECT" position at all times except during the short time period required for loading.

Automatic Injection

The most common injection system will be the automatic type. The automatic injection system uses a carriage in which multiple sample vials can be placed in position for the analysis. Load the vials with standard solutions and with filtered sample solutions. Each vial will contain approximately 4 mL. The volume to be injected is set on the instrument keyboard, as is the number of injections from each sample. The automatic system allows reproducible volumes to be injected.

Parameters needed for the automatic system:

1. Volume for each injection.
2. Set the run time for each solution. Set the run time long enough to permit the curve to return completely to the base line.
3. Number of injections for the solution in each vial.
4. Delay time between runs.

NOTE All runs can be made with the same conditions or you can set up different conditions for each sample. All runs in this series require the same conditions; therefore, only one entry of parameters is required. Set SAMPLE NO to 0 (zero) to achieve same conditions for all solutions.

ADDENDUM B

The following steps should be followed for HPLC analysis of aspirin and salicyclic acid:

1. Dry the standard or standards according to specified method.

2. Prepare the mobile phase

3. Prepare the "Diluting Solution"

4. Assemble the instrument. Put in the proper column and start flow because the column may be dirty and need to be conditioned.

5. Set up of the instrument: Set flow rate,wavelength on UV detector, and parameters on WISP and Data Module. Run mixture of Salicylic Acid and Aspirin to establish optimum conditions. Calculate resolution, relative standard deviation, and the plate count for each component. Conditions should be optimized before proceeding with the actual analyses.

6. Prepare a mixture of salicylic acid and aspirin in approximately the same concentrations.

7. Prepare the "STANDARD SOLUTION of SALICYLIC ACID" with the Diluting Solvent.

8. Prepare the aspirin "STOCK SOLUTION" with Diluting Solvent.

9. Run the HPLC analysis for salicylic acid using the optimum conditions.

10. Prepare the "ASPIRIN ASSAY SOLUTION" by diluting "ASPIRIN STOCK SOLUTION".

11. Make up the Standard Aspirin Preparation with Diluting Solvent.

12. Run HPLC on the Aspirin Standard and the Assay preparation samples.

Data Recording

Data are recorded either by a simple millivolt recorder or by a computing data module. Peak areas are measured and calculated manually when the data are recorded on a millivolt recorder. Automatic computing modules require entering several parameters for the calculations, such as:

- Chart speed
- Volume of injection
- Number of injections per vial
- Run time
- Mode (LC or SEC)
- Method of calculation
- Other parameters

ADDENDUM C

MATHEMATICAL QUANTITIES NEEDED FOR HPLC

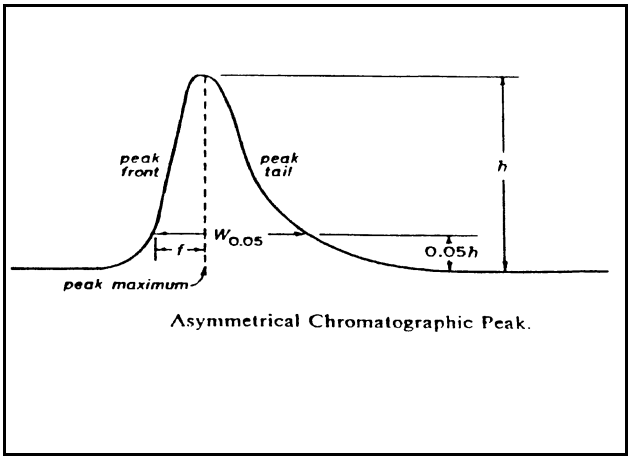
A. Tailing Factor

The tailing factor is calculated by the formula:

$$T=(W.05/2f)$$

where W = the width of the peak at 5% of the height; f = the width from the leading edge of the peak to the vertical measured from the peak.

A perfectly symmetrical peak has a tailing factor of 1.0. The USP requires this value to be no greater than 2.0. The figure illustrates how the measurements are made.



Asymmetrical Chromatographic Peak.

B. Relative Standard Deviation

The Relative Standard Deviation expressed as a percentage is:

$$S_r(\%) = (100/\bar{X})\left[\sum_{i=1}^{N}(X_i - \bar{X})^2/N-1\right]^{0.5}$$

where Xi is the area of the sample peak i and X is the average of the areas of peaks i through N in a series of N injections of the same solution. Refer to USP section (621) on Chromatography for discussion of calculations.

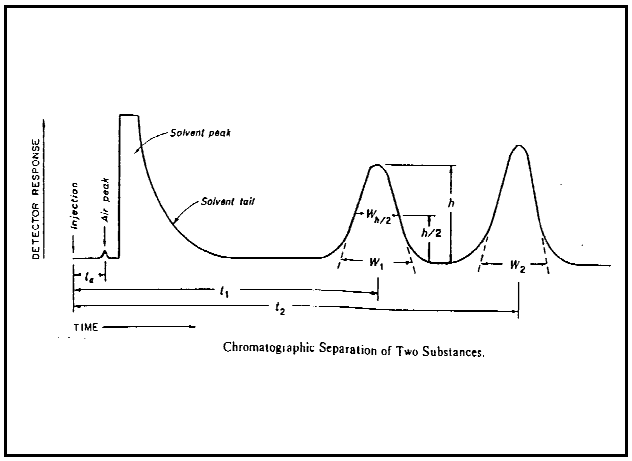
The Relative Standard Deviation is not more than 4.0% for the salicylic acid peak areas and not more than 2.0% for the aspirin peak areas.

C. Resolution

Resolution is a measure of the separation of two peaks. It is calculated from the widths and the retention times of the two peaks:

$$R = 2(t2 - t1)/(W1 + W2)$$

where W1 and W2 are the peak widths, t1 and t2 are the peak retention times, and W and t must have the same units (measure both with a ruler and express in mm). If the chromatographic parameters are proper, the resolution should be not less than 2.0. The figure shows how the resolution is determined from the chromatogram.



Chromatographic Separation of Two Substances.

D. Plate Count

Plate count is a measure of the column efficiency, and each column was supplied a plate count when the column was shipped. Normally, when a column is received at DDA, the plate count is checked by the manufacturer's and DDA's methods. These values are recorded, and any future measurement indicates the column's condition. The operator checks these values from time to time to verify the column conditions. The actual value is not important as long as you are getting a good separation of the components. The equation for plate count is expressed as:

$$n=16(t/W)2$$

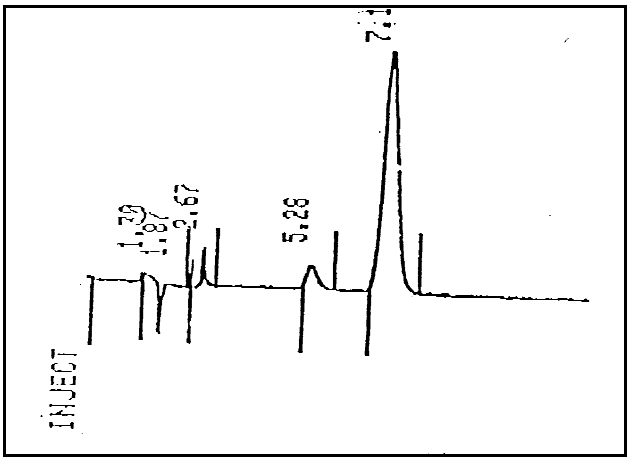
where t and W must be expressed in the same units such as mm (these measurements are made with a ruler directly on the chromatogram). The retention times and shapes of your peaks might vary slightly due to different columns, chart speeds, and other factors. The plate count should be greater than 500 plates if the separation is to be suitable.

E.Relative Retention Time

The Relative Retention Time is the ratio of the retention time for one component to the retention time for the second component as R= t1/t2

The Relative Retention Time for Salicylic Acid is 0.7 referenced to Aspirin.

ADDENDUM D



Peak at 7.11 = aspirin
Peak at 5.28 = salicylic acid
Other peaks due to solvent front
Space at middle of down side of large peak
will give the best integration

PREPARATION OF MOBILE PHASE FOR HPLC

Handling of solvents, solid chemicals, and drugs must be done with care and cleanliness. Consider all materials to be toxic. Good laboratory practices require careful handling and the use of a hood when one mixes organic solvents, such as methanol and acetonitrile. Safety glasses must be worn at all times; rubber gloves must also be worn because the materials could cause skin irritation. Buffer solutions are to be considered as skin irritants; if they contact your skin, you should wash the affected area with copious amounts of water.

Prepare all solutions in clean and dry glassware. The organic solvents must e spectoscopic grade or equivalent (impure solvents will damage the column). Water must be distilled or purified by suitable ion-exchange and filtering system. DO NOT USE WATER THAT HAS BEEN ONLY DEIONIZED BECAUSE IT CONTAINS ORGANICS WHICH WILL DAMAGE THE COLUMNS.

1. Solvents. The USP methods specify the mobile phase, which may contain buffers or modifiers. The solutions should be made in sufficient volume for the analysis. Determine the quantity needed. For example, at a flow rate of 2 mL/min, one liter (1 L) will be required for approximately 8 hours. Make sufficient solution to allow completion of the run with some left in reserve. Avoid the use of different batches of mobile phases for a single analysis. Most mobile phases used in reverse-phase liquid chromatography contain water mixed with either methanol or acetonitrile. Some mobile phases may require addition of other compounds to control ionization. Add the solvents in proper proportion to a graduated cylinder of suitable size (e.g., 1 L) and thoroughly mix. Add the modifier in the proper amount when required.

2. pH Adjustment. The USP specifies measuring the pH on the mixed solution, so you will be measuring the apparent pH. Adjust the pH to the specified pH by carefully adding the appropriate acid or base. Pour the mixture into a beaker larger than the solution volume and stir with a Teflon-coated magnetic stirrer. Use a pH meter that you have standardized with certified pH buffer solutions. Adjust the pH of the mobile phase by adding either base or acid as the case may be (USP method will specify the pH modifier). Make the additions carefully and slowly; use a disposable pipette attached to a small rubber bulb. USE CARE NOT TO OVERSHOOT THE DESIRED pH.

3. Filtering and Degassing. All mobile phases must be filtered and degassed before they are used in HPLC. Both are accomplished by vacuum filtration through an all-glass solvent-filtering system that has a sintered-glass support for a membrane filter. The membrane must be compatible with the solvent system and have a porosity no larger than 0.5 mm. For example, acetonitrile/water systems can be filtered through a Nylon membrane. Pure organic solvents can be filtered using Teflon membranes. Solvent that has been degassed will re-equilibrate with air after 12 to 24 hours. If the same solvent is to be used the next day, it must be degassed again. It is better to prepare a fresh mobile phase each day.Degassing can be done by parging with helium bubbling through the liquid. The gas is passed gently through a sintered-glass filter so that a fine dispersion of bubbles of helium passes through the solvent during the entire HPLC operation. The helium bubbles sweep dissolved air from the mobile phase, and helium itself has limited solubility. Degassing can be done by heating the mobile phase to a temperature near its boiling point. The most efficient method is the sparging, but it is also the most expensive
.4. Buffer Solutions. The USP lists the compositions for making standard buffers covering the pH range of 1.2 to 10. Silica supports and chemically bonded silica (such as the columns used in reverse- or normal-phase HPLC) can be used in the pH range of 2 to 8. Use of the support with a mobile phase whose pH is outside of this range will cause some of the silica to dissolve in the presence of strong acids or bases. The USP method specifies the pH used to control the ionization of the drug. Phosphate buffers are the most commonly used type for HPLC. Many mobile phases use buffer solutions along with the organic portion. USP analyses describe preparation of buffer solutions in terms of the 'Apparent pH' after the water and organic phases have been thoroughly mixed. Buffers of this type are made by adding a weighed amount of salt to the mixture and adjusting the pH by the addition of appropriate base or acid.

5. Gradient Solutions. Most drugs contain only one chemical

species; therefore, a gradient is not needed and isocratic conditions can be used. Some drugs can contain more than one component and may require a gradient to separate the compounds if they differ widely in polarity. A gradient system may be useful in developing an isocratic system. Each solvent in a gradient is handled as a single solvent. Each solvent must be filtered and degassed. The solvent mixtures must be compatible in all concentrations and with no precipitation of the salt, if present. To avoid the possibility of salt precipitation, DO NOT USE A 100% ORGANIC PHASE in the gradient system. Some gradients are formed by mixing two solvents, and others can be made with up to 4 solvents. The solvents required for the gradient and the rate of gradient formation will be specified.

QUANTITATIVE HPLC ANALYSIS

In all HPLC analyses you are interested in either qualitatively or quantitatively determining what is the composition of the sample. Drug analyses are primarily concerned with the quantitative aspect since you are analyzing a known drug composition. There are some occasions when the identity of the compound is not known or when no standards are available. The final step in any HPLC analysis is examination of a chromatogram to determine the amount of materials present in the sample. The chromatogram may be considered as the fingerprint of the sample contents. Interpretation of the results is most important.

Savant Tape-Slide Course 4, "Qualitative and Quantitative HPLC", has the following outline covering both aspects.

- Qualitative HPLC
  - Identification of components by retention time, "spiking"
  - Identification by spectroscopy (UV, MS, IR, NMR, etc.)
- Quantitative HPLC
  - Peak heights
  - Peak areas
  - Spiking
- Precision and accuracy
- Sampling
- Sample preparation
- Chromatography
- Integration--manual and automated
- Calculation--methods
  - Simple normalization

Corrected area normalization
  External standards
  Internal standards
  Standard addition

The Savant course covers all possible methods of calculation, ranging from manual to computer integrated and calculated. Since manual calculations are seldom done, the discussion here will emphasize the digital electronic method using areas under the peaks in the chromatogram. It is wise, however, to know how the computer makes its calculations of the data. Areas can be measured manually by triangulation, cutting and weighing, and planimeter. Some measurements are possible using peak heights, but this method does not consider any change in the chromatogram due to peak spreading. As a result some error is introduced. Peak areas give better accuracy in most cases. The chemist must analyze the chromatogram and determine if the data look correct or not.

SOURCES OF ERROR

Errors can occur in many places along the many steps, so be aware where these errors occur and how to avoid the pitfalls. The following possible errors may be found in HPLC operations:

(1) Sampling--material not homogeneous. In all cases the sample must be representative.
(2) Sample preparation. Samples must be prepared with the best analytical procedures.
(3) Loss of sample.
(4) Leaks in HPLC system.
(5) Overlapping or undetected peaks.
(6) Detector errors.
(7) Recorder errors.
(8) Errors in base line correction.

Any one of the above errors or combinations of these will result in unreliable data.

There are two important properties in HPLC analysis, namely: Precision and Accuracy. Precision is a measure of how

reproducible your data may be. Accuracy is the measure of the true value. You can have excellent precision but poor accuracy. Precision is measured as the standard deviation, which when expressed as a percentage of the mean is called relative standard deviation. The standard deviation or relative standard deviation gives you the spread in the data; both are measures of the repeatability of the measurement. One component of accuracy is the linearity of instrument response versus amount of drug; injecting twice as much of the drug should give twice the response. A measure of linearity is the correlation coefficient, which is one of the results obtained from a "linear regression" analysis.

Measure the plate count and the resolution of the column as described in the HPLC Training Module. Determine the retention time of the nonretarded component, t_0, on your column. This retention time, in minutes, multiplied by the flow rate, in mL/minute, gives you the volume of liquid in the column, also called the "void volume" (the t_0 needs to be determined only once, as it is independent of solvent). Nothing in the sample can elute before time t_0. The t_0 is determined by injecting some compound which is not retained. A dilute solution of NH4NO3 in water serves as a good measure for t_0 in C18 columns.

HPLC can only be used to measure compounds quantitatively after calibration of the instrument. The USP uses two general methods to calibrate, namely, internal and external standards.

INTERNAL STANDARDS

Internal standards are used to compensate for variations in injection volume, flow rate, and temperature. Internal standards must fit certain properties as follows:

a. Substance must not be found in sample.
b. Peak must be well resolved from the sample peak.
c. The standard must be available in pure form.
d. Gives a detector response in the same range as that given by the sample.

Develop a calibration curve as follows:
Run a series of standard solutions which contain known, different amounts of pure drug and a constant amount of internal standard. From the chromatogram of each standard solution calculate the ratio

Rs = (area of drug/area of internal standard).

Plot Rs on the Y axis and concentration of pure drug on the X axis. The slope of this plot, R = (Rs/amount), is the response factor.

EXTERNAL STANDARDS

The external standard is the same substance as that being assayed in the sample. The USP uses this method of calibration in most cases. The external standard must be pure or the composition known through prior analysis. Many times the standard is costly or in short supply so a secondary standard is used. The concentration of the standard solution is kept near to that of the sample solution to avoid errors. The areas of the corresponding peaks in the standard and the sample chromatograms are compared using simple ratios. The External Standard method reduces errors due to response, nature of sample, variation in concentrations, etc.

Develop a calibration curve of the area versus concentration of the external standard. Use the peak area on the Y axis and concentration on the X axis. The slope of the plot is the response factor. The amount of the component in the sample is determined from the curve. Digital electronic integrators read numbers to 5 to 6 places. Do not report results to this many significant figures because inherent errors in the measurements do not warrant them. Normally, one uses four significant figures in the calculations and rounds off the reported result to three significant figures.

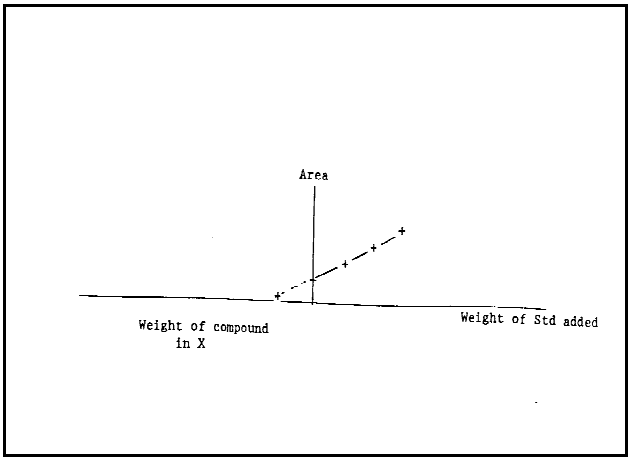
There is another calibration method which can be used in some cases where quantitative sampling may be a problem.

STANDARD ADDITION METHOD

Sometimes it may be necessary to determine the quantity of a substance where sampling is a problem. A method known as STANDARD ADDITION or SPIKING can be used. In this method we inject several solutions. One of them contains only the sample, but the rest contain sample plus added known amounts of standard. Let the unknown amount of drug in the sample be represented by X. Let this amount be zero on the X axis. Add successive quantities of the standard to the original to form X + W1, X + W2..... X + Wi. Plot the areas versus weights of standards added. Extrapolate back to zero area by a least-squares fit of the data. The negative intercept on the X axis will be the weight of the

compound in the original sample. The drawing below shows how such a plot would look:

The attached curve from an actual HPLC analysis of a main component and a trace substance found in aspirin show how the results are calculated. In this case the impurity is known and a standard available. The impurity can be reliably measured.



TRACE ANALYSIS

Many samples that are analyzed by HPLC are not pure and require determination of impurities which may be present in low levels. When the concentrations are low or the response factor is low, the impurity may not show up clearly on the chromatogram used to assay the sample for the main component. In other cases the absorptivity of the impurity may be high or the impurity may be present in large amount; if so, the chromatogram will indicate the presence of other substances. If the impurities elute at the same point as the main substance, you cannot measure the trace material. If the trace material and the main compound are separated, a quantitative measurement can be made on both. In order to measure the trace material it is usually necessary to make two different concentrations of the sample. A dilute solution is made for the assay of the main component and a concentrated solution for the trace analysis. The concentration for the trace analysis may be in the order of 10 times greater than the concentration used for assay of the main component. When HPLC is run on the concentrated solution, the peak for the main component will be off scale. Ignore the main peak and measure the area of the trace material. Most times the nature of the impurity will not be known, so you assume that the response to the impurity is nearly equal to that of the principal component. Carefully record all concentrations so that you can calculate the percentage of the trace in the original sample.

DRAWING BASELINES

A baseline is generated during a run and represents the signal with only the mobile phase going through the system. When the baseline is linear with zero slope and the peak is clearly defined, draw the baseline by connecting the two points where the peak begins and ends. When baselines drift then some problems arise from deciding where the actual baseline is. In most automatic systems, the analyst can choose where to draw the baseline. When there are overlapping curves, the computer draws a line perpendicular from the point of intersection to the base resulting in error. The computer normalizes all the areas and reports the percentage of each component without regard to any difference in response unless the program has been told to adjust on a response factor. To do this, measure each component for response. Most reliable data are obtained from a corrected normalized curve.

HPLC COLUMN CARE

The HPLC column is the most essential part of the system. All columns have a limited life, and the proper care of the column will determine the useful time. Chemists like to keep and maintain their own columns because they know the history. There are certain procedures which aid in keeping a column in good condition.

TYPES OF COLUMNS

The USP lists columns according to the types of packing used in drug analysis. The list can be separated into two general types, namely: (1) nonpolar packing for reverse-phase separations and (2) polar packing for normal-phase separations. Most drug analyses are done by reverse phase with coatings such as C18, C8, or phenyl. For example, the most widely used is a L1 packing, which has octadecyl silane chemically bonded to porous silica or ceramic microparticles whose diameters are from 5 to 10 mm. The L3 normal-phase column packing is porous silica. The USP method specifies the type and size of column for each analysis. SELECT THE CORRECT COLUMN for your specific analysis.

INITIAL INSPECTION OF COLUMN

All HPLC columns come prepacked with a solvent suitable for long-term storage. Each column is supplied with a measured plate count and method of testing. The column should be tested according to the instructions to determine initial quality and to serve as a basis for checking column performance. Such a check is the only acceptable means to determine if the column meets specifications.

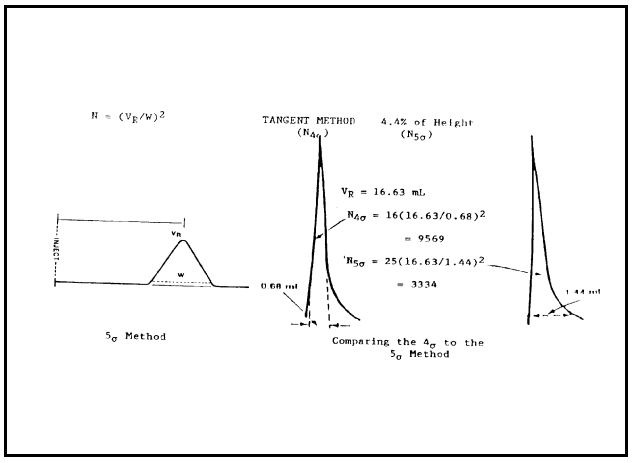
1. Connect the column in the HPLC system with the flow in the direction marked on the column. Eventually the column will be connected to the injector and the detector. Initially leave the output of the column disconnected while you are changing solvents. CAUTION. Columns are furnished with different types of end fittings. Make sure that the fittings on the column and the HPLC instrument are compatible in order to make a leak-free connection. The critical factor is the distance between the ferrule and the end of the tubing. Columns are connected and disconnected many times over their lifetimes, so each time an additional pressure must be applied to make a leak-free seal. Eventually the fittings will be damaged and must be replaced. Universal plastic fittings compatible with most columns are available to permit seals to be made with finger-tight pressure,

which will increase the lifetime of the end fittings. Plastic fittings made from PEEK(polyether ether ketone) resin are recommended. Such fittings are compatible with most HPLC solvents.

Columns should be handled carefully without mechanical shocks or vibrations and kept free of wide temperature changes.

2. Start the flow at 0.2 mL/min and gradually increase the flow. Replace the original solvent with the desired solvent system. When changing solvents the flow must always be low to avoid any sudden change in pressure. When the desired mobile phase is in the column and you have obtained a steady, noise-free baseline, you are ready to check the column performance.

3. Plate Count and Resolution. There are different ways to measure plate count. Two ways of testing seem to used, namely (1) the tangent method and (2) the five sigma method. The methods of calculation are shown below:



The tangent method assumes the peaks to be symmetrical (e.g., gaussian shaped) and uses the relation n = 16(t/W)2, where t is the retention time and W is the width of the peak at the baseline expressed in the same units. The simplest and most accurate method for measuring plate count is to measure the retention time and width with a ruler and express both in terms of millimeters. The USP uses the tangent method for expressing plate count.

In the five-sigma method, we assume the peak is gaussian; therefore, we could compute a "standard deviation" (sigma) from the distribution of the peak. In practice, the computation is made much simpler by assuming that five standard deviations (five sigma) are encompassed by the peak width measured at 4.4% of the peak height. The plate count is expressed as n = 25(t/W)2. The five sigma method gives values about one-third those of the tangent method. This method takes into account tailing in the separation and gives a measure of column performance. The method is subject to error due to the position of measuring the baseline. Both methods of calculation can be made on the same retention peak. You should try both methods initially. Select the USP method as the one to use, and record the values for future reference.

4. Plate Count. Waters C18 Columns contain 70:30 mixture of methanol:water when shipped. Replace this solvent with a 60:40 acetonitrile:water mixture. Set the flow rate at 2 mL/min and inject a sample containing uracil and acenaphthene. The uracil is unretained and the acenaphthene will be retained for a considerable time. The uracil will give a direct measure of the void volume of the column. The void volume is used to estimate the volume of solvent needed to clean or regenerate columns. Check value with supplier's value. The method and compounds listed in this paragraph are those suggested by Waters Associates for measuring plate count on their C18 columns; when using columns from other suppliers, follow their recommended methods. Another method for measuring plate counts and resolution is the use of a standard mixture designed to fit the type of operations normally in your laboratory. A solution of the following compounds is used to establish column characteristics for drug analysis: 50 mg of p-hydroxybenzoic acid

200 mg of propyl paraben (propyl ester of 4-hydroxybenzoic acid)

150 mg of ethyl paraben (ethyl ester of 4-hydroxybenzoic acid)

250 mg of n-butyl p-aminobenzoate

Dissolve the compounds in 25 mL of acetonitrile. Dilute 1 mL of this solution with 25 mL of 50% acetonitrile.

NOTE--These weights do not have to be exact as you are only interested in a good response.

Prepare a mobile phase consisting of 40:60 acetonitrile:water modified with 0.2% phosphoric acid. Set the flow rate at 2 mL/min. Set the UV detector at 254 nm. Measure the void volume by injecting about 5 mL of a solution of NH4NO3 in water (4 mg/mL). (The void volume will be essentially the same for

all solvents).

5. Inject about 10 mL of the solution containing the four test compounds prepared earlier. Calculate the plate count and the resolution from the chromatogram. Checks on plate count and resolution should be done at regular intervals or as a means of detecting column problems.

SOLVENTS AND SAMPLES

1. Solvents. All organic solvents must be distilled in glass and spectroscopic grade or better. Impure solvents will damage the column. Water must be freshly distilled or purified by a suitable system that includes filtration, deionization, and charcoal treatment. DO NOT USE DEIONIZED WATER because it contains organic materials which will damage the column. The pH must be kept in the range of 2 to 8. Strong acids or bases will cause some dissolving of the support. Do not use water alone as the mobile phase. Use a buffer to maintain a pH of 7.

Filter and degas the mobile phase by vacuum filtration before pumping it through the column.

2. Samples. If samples are dissolved in a liquid other than the mobile phase, the liquids must be compatible. Sample solutions must be free of any turbidity.

3. Guard Column. A very short column is placed at the entrance to the analytical column to protect it from insolubles. The guard column contains the same type of packing material as the main column. The packing is replaced when the guard column becomes plugged. Disposable cartridge columns are available and are often used.

CARE OF OPERATING COLUMN

1. Column Flushing. Most mobile phases used in drug analysis contain some form of salt as a buffer or ion modifier. Salt of any kind will damage the columns, pumps, and detector if allowed to remain in the system. DO NOT LEAVE SALT OF ANY KIND IN THE COLUMN WITH NO FLOW FOR ANY PERIOD OF TIME (such as overnight or weekends). The column must be cleaned of salt when flow is stopped overnight or for longer storage. Salts will cause corrosion and reduce resolution. When salts are in the column, never go initially to 100% organic phase as precipitation may occur in the column. Wash out any salt with purified water, and then go to other solvents. The following steps outline the procedure:

1. Flush with a solution similar to the mobile phase but with salts removed (five column volumes).
2. Flush with organic solvent used in the mobile phase, for example, 100% acetonitrile or methanol (10-20 volumes).
3. If step (2) is not adequate to remove salts, flush with 10 to 20 volumes of methylene chloride.
4. Flush with straight organic solvent (10 volumes) as in Step 2.
5. Flush with nonbuffered phase.
6.Return to original mobile phase.

As a rule of thumb, the standard 30-cm column will have a void volume of approximately 3 mL, so 10 volumes would be 30 mL. The column void volume that you determined while you were measuring the column plate count and resolution is a better value to use. The flow rate can be increased to reduce time of flushing. Do not increase the flow past the point at which the column's upper pressure limit is exceeded. The volume is the key to successful flushing. Do not increase flow to the point where column pressure limits are exceeded. When changing solvents, always start with low flow and gradually increase it.

Alternate to flushing. Keep continuous flow of the mobile phase at 0.1 mL/min overnight. Next day replace the solvent with freshly made and filtered mobile phase. Do not use the old solvent as it will have reached equilibrium with air and bubbles will form in the column. If it is necessary to use the old solvent, degas it again.

RULE OF THREE

A good rule to remember is the "Rule Of Three", which says that a change of 10% in the mobile phase will alter the retention time

by a factor of three.
Use this as a means of adjusting the mobile phase to get better retention times.

COLUMN STORAGE

Short Term (overnight or weekends). Flush column free of salts and store it loaded with a solution similar to the mobile phase being used but with salts omitted. Equilibrium will be quickly reached when you restart with the buffered mobile phase. Do not store a column filled with water alone. Alternatively, one could store the column filled with the organic/water mixture in which the organic solvent is present at greater than 50%, but longer times to reach equilibrium will be required on restarting.

Long Term. Flush the column free of all salts with 10 to 20 column void volumes of pure water. Select a storage liquid: Use the liquid recommended by the column supplier (the original shipping solvent); if you do not know which solvent the supplier recommends, use a 70:30 mixture of methanol: water. Pump 10 column volumes of storage liquid through the column. Remove the column from the HPLC system and tightly cap both ends to prevent dryout.

COLUMN PROBLEMS

1. PRESSURE BUILDUP

The column comes with a recommendation for the maximum pressure that is advisable for its operation. The pressure should be noted when the column is initially put in service. Pressure buildup is caused by accumulation of contaminants or insoluble materials. Careful filtering of sample solutions and mobile phases will prevent pressure buildup most of the time. Use of a guard column will also aid. Most likely the buildup will be located at the entrance end of the column. The easiest thing to do is to reverse the flow in the column. (Some columns will be damaged by reverse flow; read the manufacturer's instructions before reversing the flow). Disconnect the column and reconnect only to the pumping system. Do not pass the stream through the detector. If pressure has not been reduced when you retest the column, remove The end fitting at the column entrance end and check the stainless-steel frit. Either replace it with a new frit or clean the old one by sonication in a dilute solution of nitric acid.

2. LOSS OF RESOLUTION

Loss of resolution of the peaks shows up as lower plate count and spreading of the bands. Peak height will also be decreased. Resolution loss is usually caused by buildup of nonpolar compounds which are soluble in the silane phase of the packing. Sample precipitation will also cause resolution loss. If at some time the pH was out of the range of 2 to 8, some of the packing may have dissolved, leaving a void at the column end. Any of these causes alter the resolution. Check the resolution you originally obtained when you first installed the column. If the cause is due to buildup of contaminants, the column can be regenerated. Follow the procedure as outlined:1. Contaminants with polar groups. Wash the column with 25 mL of pure water; follow with 50 mL of methanol. During the methanol wash make four or five 2-mL injections of dimethylsulfoxide, using manual injection.

Contaminants with nonpolar groups. Wash with 50 mL of methanol, then 50 mL of methylene chloride, then 50 ml n-heptane, and return to polar solvents by washing with methylene chloride and then 50mL of methanol. Before starting this procedure, clear the column of any salt. If the regeneration was not effective, replace the column as soon as possible.

The procedures outlined are applicable to C18 columns. Other columns will have their own procedures for operation. Consult the supplier's data on the particular column. Proper care and handling of columns will greatly extend their lifetime and performance.

HPLC TROUBLESHOOTING

HPLC is a widely used method for drug analysis, and the success of an analysis depends upon proper functioning of all component parts. Since the operation of HPLC depends upon mechanical and electronic parts, it is important to recognize when the instrument is not functioning properly and to locate and repair the malfunction. Certain symptoms will locate the malfunction and lead to rapid diagnosis. The chemist can make some simple repairs which will keep the system in operation with minimum delay. Most actions described are classified as preventive maintenance and should be performed regularly.

The Savant Training course available at the FDA provides useful informationon troubleshooting in HPLC. Savant Tape-slide course 5 "Trouble Shooting in HPLC", outlines problems that occur. The course is presented in three parts as follows:

Part 1
Malfunctions in Pump, Injector, Detector and Recorder.

Part 2
Malfunctions in Column.

Part 3
Detecting Malfunctions from Chromatogram.

Most problems in HPLC will be found in either the pump or the column and generally are flow related. A large number of problems can be prevented or reduced by carefully filtering and degassing the mobile phase after preparation. A filter should be placed on the end of the supply line in the solvent reservoir. All samples should be filtered through a membrane filter having a pore size no greater than 0.45 mm. The analyst needs to recognize when the chromatogram is not correct and when the data are reliable. Problems are of two kinds, namely:

(1) Those that the chemist can correct with little effort
(2) Those that require experienced service personnel for extensive maintenance.

This training module will emphasize problems the analyst can correct with minimum downtime and will help the analyst describe other problems to the service department.

COMMON PROBLEMS IN HPLC

PUMPING SYSTEM

(1) HIGH PRESSURE. High pressure will be experienced at some time in all HPLC operations. A buildup of impurities on the column or filters will cause increased pressure. See the "HPLC COLUMN CARE TRAINING" module for specifics on cleaning and restoring columns, and cleaning of filters. High pressure will also be observed at high flow rates or when changing solvents having different viscosities. For example, the methanol/water system is notable in exhibiting higher pressures when the concentration of methanol is increased. Refer back to the pressure observed when the column was first installed so you know when a change in pressure has occurred. Reducing the flow rate will lower the pressure, but will increase the retention time. During the entire use of the column, keep pressures within the recommended limits. Set the pumping system to shut off before the maximum pressure for the column is reached.

High pressures can be caused by plugging in the pump, injector, supply lines, column, and detector. First determine the location of the blockage by working backwards beginning by disconnecting at the detector and continuing backwards to the pump by removing one part at a time. The most frequent blockage point will be found at the entrance frit of the column.

(2) NO PRESSURE. No pump pressure will be observed when the pump cannot draw solvent on the inlet side, as might be caused by plugging in the solvent inlet filter located in the solvent reservoir or a malfunction of the solvent draw-off valve. The solvent draw-off valve and manifold can be checked in the same manner as priming the pump. If the valves are not working, the solvent from the syringe will flow back into the solvent reservoir with only a slight applied pressure. If either the pump inlet or outlet valves of a single pump are stuck in any position, pulsing flow will result, causing the pressure to fluctuate. When you have flow and still no pressure, then the pressure transducer is the problem and will need to be replaced. NOTE--The column or some other form of flow resistance must be connected to the pump to detect pressure changes.

(3) FLUCTUATING PRESSURE. Fluctuating pressure will be observed when one piston head is pumping and the other not functioning. Air bubbles entrapped in the valving system will cause one piston head to not function, giving a cyclic pressure output. Try

repriming the pump with degassed mobile phase; use a 10-mL gas-tight hypodermic syringe attached to the draw-off valve and force liquid up into the outlet valves. Continue to apply the pressure on the syringe while the pump is operating. If the pressure remains erratic after several attempts to prime the pump, disconnect the column from the system. Flush out the salt solution with deionized and filtered water, then with methanol, and finally with isopropanol. Sometimes the pump outlet valves stick because of accumulation of materials on the surfaces, and isopropanol will usually remove the impurity. The whole system can be cleaned with isopropanol at high flow rates (BE SURE THAT THE COLUMN IS NOT CONNECTED AND THAT THE SYSTEM IS FREE OF SALT). The pump will need to be primed each time that it is restarted after a prolonged downtime.

(4) SOLVENT LEAKS. Leaks in any part of the HPLC system will cause erratic flow, and retention times or areas will not be correct. All connections in the system must be leak free. System leaks are due to: 1. fittings not tight, 2. noncompatible fittings, and 3. damaged or scored fittings. Make sure you have the proper fittings that are compatible with your system. If you don't, you can never stop the leak. All connecting tubing must be the proper size (0.009 inch ID) leading from the injector to the detector. Try first tightening the fittings using the proper sized open end wrench. Do not use adjustable wrenches to tighten fittings. Over tightening can damage fittings and cause leaks. If any fittings have been damaged, new fittings of the same kind must be installed.

Leaks around the back of the piston indicate worn or deformed plunger seals and possible plunger scoring which can be caused by excessive wear or by salt deposits. When salts are deposited in the pump the sapphire plunger will be scored or show excessive wear. Regular flushing of the entire system before the HPLC is put in stand-by mode will prolong the life of the seals. The seals in both pumps are replaced by removing the pump head. (If you are not familiar with changing seals, contact the service people or look in the service manual for directions to change seals.) BE SURE TO DISCONNECT THE COLUMN FROM THE SYSTEM BEFORE STARTING ANY FLOW TO BREAK IN SEALS. When replacing pump seals, it is a good idea to replace seals on both pumps since both probably have equal wear. When seals have been replaced, prime the pump with methanol and start the pump flow at 0.2 mL/min (DO NOT START THE PUMP AT ANY HIGHER FLOW RATE). Gradually increase the flow rate to break in the seals and continue until everything runs smoothly. Avoid the use of halogen-containing salts whenever possible. If halogen compounds cannot be avoided, the

life time of the seals will be reduced and more frequent replacement will be required. Also, halogen compounds can cause corrosion of the 316 stainless steel in the system.

DETECTING MALFUNCTIONS FROM THE CHROMATOGRAM

Many of the common problems in HPLC will show in the chromatogram since the response will be dependent on the detector, column, and pump. You need to be able to recognize problems quickly. Problems related to changes in the column plate count and resolution will show as change in the retention time, peak spreading, or both. See the "Training Module on Column Care".

1. The samples can also change the shape of the chromatogram. If some of the samples contain materials which are strongly adsorbed on the support, the curve will show tailing (curve changing from gaussian to a distorted shape). Cleaning or possible regeneration of the column is required.

2. If the leading edge of the chromatogram rises slowly, the column could be overloaded. Reduce the volume of the injections and/or concentration.

3. Sometimes a single peak will degenerate into a double peak or one with a shoulder. A dirty column or inlet frit will cause such behavior.

4. All peaks of the chromatogram are caused by some material eluting from the column and must be considered. The chemist needs to know when to accept or reject the data. Any peaks other than those expected from the injected compounds could be due to solvent, anions such as maleates, isomers or artifacts. Repeat the injection and examine the reproducibility. Impurities will show up in the repeated chromatogram at the same retention time and magnitude. Artifacts arise from previously adsorbed materials, and the positions and heights of the peaks occur at random. If the impurity peaks are separated from the peak of interest, such peaks can be ignored in the calculations. Complications arise when impurities show up under the main peak or as a shoulder or shoulders on the peak of interest. Some change in the mobile phase may be necessary to attempt separation of impurities from the principal component. Unless the impurities can be separated from the main compounds, no determination of impurities can be made.

5. The baseline of the chromatogram must also be linear with zero slope. Solvent changes, partially retained compounds, or a dirty

detector will cause the baseline to shift. Continue the flow until a steady, noise-free baseline is established. Baseline noise can be caused by bubbles, contaminants in bad solvents, contaminated column, the detector cell, leaks in the system, erratic flow, or electrical problems related to the recording system. The HPLC analysis cannot be continued until a suitable base line has been established. Never depend on the first chromatogram in a run. Use this trace to adjust sensitivity, flow rate, sample size, or other parameters needed to produce a reliable chromatogram. Replicate all samples unless otherwise instructed.

Many troubles in HPLC can be eliminated or reduced by flushing the column and instrument with water and finally with the methanol/water mixture. See "HPLC COLUMN CARE" module.

SECTION IV

GAS-LIQUID CHROMATOGRAPHY

INDEX

GAS CHROMATOGRAPHY TRAINING MODULE

Gas chromatography (GC) is a tool well suited to certain types of drug analyses. It provides a rapid analysis of a variety of organic materials. The method can be applied only to those compounds that can be vaporized without degrading at the elevated temperatures required. Gas chromatography is extremely accurate for measuring low molecular weight compounds and detecting impurities at very low levels. Because of the instability of organic compounds, the method is applicable to only about 15% of the total number of compounds. There are two criteria for any sample to be analyzed by GC, namely:

(1) the sample must be thermally stable and (2) the compound must have sufficient vapor pressure and must be capable of vaporizing.

Training Courses

The FDA laboratory has available materials which can help you learn or review the essentials of gas chromatography. Even if you have had prior experience with this technique, you may find it profitable to spend some time with written, audio, and visual instructional materials. Tailor your instruction to your personal needs. Each course will take approximately 1 hour to view and study. Spend as much time as necessary.

SAVANT Tape-Slide Course #1, "Basic Principles of GC-Instrumentation
- Components"
- Carrier gas supply and regulator
- Sample inlet system
- Column in a controlled temperature oven
- Temperature controlled detector
- Recorder or data analyzer
- Electronics for control

SAVANT Tape-Slide Course #2, "Trouble Shooting"
- Electronics
- Application problems
  - Flow
  - Decomposition of sample

SAVANT Tape-Slide Course #3, "Qualitative and Quantitative Analysis by GC"
- Qualitative

Quantitative
Sample
Sample preparation
Digital conversion
Calculations

SAVANT Tape-Slide Course #4, "Capillary Columns"
Types of capillary columns

NOTE--The portion of the Savant course on capillary columns should be postponed until after you have some experience with GC analyses. Capillary columns are a means of extending the sensitivity of the analysis.

PREAMBLE TO LABORATORY PRACTICES

The GC operation requires that gas be handled in high pressure cylinders, and that equipment be operated at elevated temperatures. Careful planning is required to ascertain the operation and proper supply of gas. All gas cylinders must be properly secured to the laboratory bench with a suitable chain or strap. Any movement of gas cylinders from the storage area to the instrument must be performed with a cylinder-handling cart. Safety glasses must be worn at all times and insulated gloves must be available if hot surfaces are to be handled. You should keep in mind that many of the surfaces of the instrument are operating at elevated temperatures, and can cause burns. The instrument must be cooled before any changes in the heated areas are attempted. Also, the gas supplies must be shut off during any changes involving opening gas lines.

Many of the GC instruments are equipped with flame ionization detectors that burn with a mixture of hydrogen and air. You should keep in mind that hydrogen gas is very flammable and explosive. All connections must be free of leaks; if any leaks are found, they must be corrected immediately. The hydrogen should be left on only during the GC runs and not during extended idle time.

Most drug analyses specify the use of glass columns, which are fragile. The columns are made to fit specific instruments, so no attempt should be made to force any column to fit into the system. Care must be used in handling to prevent breakage and possible personal injury.

Many of the analyses require extended preparation of the sample or standard, such as drying and extraction. Standard samples should be obtained from the laboratory supply source and dried according to directions supplied on the label. The dried material should be stored in a desiccator until ready for use and kept stored until the analysis is completed. Your analysis should be planned to include all the necessary preparations. All GC operations require elevated temperatures; time must be allowed for the instrument to come to equilibrium. In many cases, the sample can be prepared while the instrument is coming to equilibrium.

INTRODUCTION

Before a gas chromatography analysis can be made, the components must be assembled. The instrument itself contains the ovens, detectors, and injectors. Instruments are made so that different types of operations can be performed by making changes in detectors, types of columns, and injectors. The USP method specifies the type of column, carrier gas, and detector to be used. The recommended temperature and flow rate are given. With this information, the GC system can be arranged as specified.

Most drug analyses operate with the gas as the mobile phase and a liquid as the stationary phase; therefore the type of separation is known as GLC (gas-liquid chromatography). Packed columns are used for separations. The columns usually are glass tubing about 6 mm OD by 1.8 meters (in English units, about 1/4 inch OD by 6 feet long), coiled to fit the specific instrument. Columns with this outside diameter are available in 2, 3, and 4 mm ID. The columns are packed with an inert support coated with a high-boiling liquid. The liquid coating used will depend upon the polarity of the compounds to be separated. The type of column specified by USP XXII should be selected. Each GC instrument manufacturer has a special geometric configuration for the column; therefore the column made for the instrument must be used. (NOTE-- The jet to the detector must be checked before the column is installed.) The jet must be suitable for a packed-type column. (See Addendum A under Operating Instructions for HP 5890A GC.)

Glass columns are fragile and will break if strained. In order to avoid breakage, the column should be connected to the injector and detector at the same time with a torque wrench. The glass column is connected to the metal fittings of the instrument by means of a 1/4-inch nut and ferrule or O ring. A graphite ferrule is preferred, as it will be suitable over a wide temperature range. Some Hewlett-Packard instruments are equipped to handle column coils 9 inches in diameter, while others use 6-inch coils. It is possible to use the 6-inch coil in instruments having space for the 9-inch provided a suitable adapter is used. You will not be able to use the 9-inch coil in instruments designed for the 6-inch column, however. Column connections are made with a special torque wrench to prevent too much tightening, which can result in column breakage.

Most GC instruments have the capability for two detectors, and

you must choose the detector specified. The USP methods use flame ionization unless otherwise specified. The flame ionization detector (FID) is specified in this training module. (Some methods call for thermal conductivity or electron capture for the detector.) When the FID is used as the detector, the sample is burned in a flame from a mixture of hydrogen and air, converts the carbon compounds to ions that are captured by a collector to produce an electric signal proportional to the number of ions formed. The FID system is very sensitive, but cannot be used to detect permanent gases or water. Compounds containing chlorides or silicon can cause damage to the FID because of corrosive compounds formed and silica deposits. Continued use of such compounds will require repair or replacement of the detector. The outlet from the column isconnected to the FID.

After making the column connection to the proper detector and injector, you are ready to arrange the gas supplies. The carrier gas will be either purified nitrogen or helium. FID has greater sensitivity with nitrogen, but helium is a good substitute. The gas is connected to the injector of the instrument through a regulated flow meter. The FID uses a mixture of hydrogen and air for the flame; both gases are supplied through a carefully regulated system. All leaks must be eliminated. After the gas connections are made, the system should be tested for leaks with a leak test fluid, such as "SNOOP", by brushing some of the liquid around the connectors. (Also see Operating Instructions for HP 5890A GC.) Bubbles will form if there are any leaks. After testing for leaks, you should wash away the SNOOP so that no deposit will be left. Carrier gas flow must be regulated and constant throughout the runs, if the retention times are to be meaningful. The flow rate recommended by the USP should be considered as a starting point; at the same time it should be recognized that different columns could have different flows and thus require some adjustment for maximum efficiency. The flow should be altered to achieve the best operation of the system.

There are two or three injection techniques. In the most common type, the sample is injected by a method known as "direct addition to the column". In this method the cool liquid is injected directly onto the head of the column that sits inside the injector oven. The sample is vaporized and immediately starts its path through the column. This causes the peaks to be sharper and reduces tailing. The sample is drawn into a microliter syringe to which a needle is attached (the needle may or may not have a valve to permit injection of gas samples). The injector contains a flexible rubber septum, and the sample is injected onto the column through the septum. The septum is

partially damaged each time an injection is made, and must be replaced before beginning a run. After the septum has been used for several injections, leaks will develop; the septum must be replaced when this occurs. A bent or damaged needle causes excessive damage to the septum, and should be replaced with a new needle having a sharp point and no ragged edges.GC can be run in an isothermal condition, in which the temperature remains constant throughout the run, or by a temperature gradient. Temperature gradients are used when the components have widely separated boiling points or different solubility behaviors in the liquid phase (large difference in the polarity of the compounds). A gradient is achieved by setting an initial temperature, a rate of temperature rise, and a final temperature. When gradient temperatures are used, the instrument must be allowed to return to the initial starting temperature before the next sample is run. Systems operated by a controller or computer will automatically adjust to the initial starting temperature before the next sample is injected.

Carrier gas flow is measured by a glass-bubble flow meter or an electronic meter. The gas is measured as it exits from the detector, before the detector is lighted. If you are using a bubble flow meter, a flexible rubber tube is connected between the outlet of the detector and the flow meter. A bubble is introduced into the glass flow meter by lightly squeezing an attached small rubber bulb and forcing an air bubble from a soap solution into the flow meter (a dilute soap solution will form the bubble). The glass tube has graduations with a lower and upper volume marking similar to a pipette. Flow is determined by measuring the time it takes the bubble to rise from the lower mark on the meter to the upper mark, using a stopwatch.

Some GC instruments are equipped with an electronic flow sensing meter, and the flow rate will be displayed automatically. Corrected flows for the different gases are defined by keyboard entry. A direct reading of the flow is displayed when called by the keyboard.

Before beginning the laboratory exercise below, you should have developed the volumetric and gravimetric skills typically required in the preparation of a sample for GC. If you are not confident that your skills in these techniques are adequate, you should stop here and review those procedures or learn them afresh.

The gas chromatograph is now assembled and you are now ready to set temperatures flow rates for maximum efficiency, and to

measure the column characteristics. New columns must be conditioned by flowing the gas through the system until a stable base line has been established.

SETUP AND TRIAL RUN

(Refer to Operating Instructions for HP 5890A GC for additional information while using this section.)

Any column, whether it is used or new, may contain contaminants and must be conditioned before analytical use. New columns may also contain volatile contaminants which must be driven off. CONDITION THE COLUMN WITH THE FLAME LIT. The preferred carrier gas for conditioning is He, but N2 can be used. DO NOT USE HYDROGEN because the gas will exit directly into the hot oven.

OLUMN CONDITIONS:

1. Flow rate 15-40 mL/min helium. (NOTE--The USP uses a 3 mm column but you will be using a 2 mm column, so the flow isreduced. You will need to establish the best conditions.)

2. Oven temperature: Set at 260EC.

3. Injector and detector temperature: Set at 265EC.

To condition the column, use a 265EC oven temperature for 1 hour; then cool the oven to 260EC. Do not exceed the 265EC.
After the flame is lit and the chromatographic/integration conditions have
been set (see ADDENDUM B), prepare a solution of Testosterone Cypionate secondary standard at the concentration specified in the USP XXII. Make a 1 mL injection, and press START RUN on the integrator. Observe the peak obtained and the baseline; the baseline should not drift substantially. The peak should elute within about 10 minutes. Check the integration marks and the shape of the peak. If the peak is too small or too big, increase or decrease the attenuation of the signal and/or the injection volume as appropriate. Change the attenuation on the HP 3396 integrator to lower numbers to increase the peak signal. If the integration marks are not correct, check the HP integrator manual for guidance on correcting integration of a peak. Check to see if the THRESH (threshold) and PKWD (peak width) need changing. The helium carrier gas flow may also have to be made faster or slower.

Repeat the injection to optimize the chromatogram; then prepare a solution of the internal standard, and inject it. The retention

times of the internal standard and the sample must be different so that the two materials separate clearly. An internal standard with similar chemical structure is also desirable. Observe the retention time, peak shape, and integration marks; make sure no peaks or interference is obtained in the region of testosterone cypionate. When conditions are suitable, prepare a mixed solution of testosterone cypionate and internal standard at the concentration specified in the USP XXII procedure, and inject it. Optimize the conditions to achieve correct integration if this is needed.

Make six injections of the mixed standard solution. Discard the first injection and calculate system suitability, based on the calculations of coefficient of variation (relative standard deviation), resolution, and tailing factor as described in Addendum A. Record all the conditions used in the analysis on your worksheet. Press LIST,LIST on the HP 3396A integrator (see Addendum B) to record the integrator conditions.

When the system suitability criteria are met, you are now ready to start the USP analysis. (See Addendum A for suitability limits.) The USP describes the analysis of testosterone cypionate injection (USP XXII, page 1327) by GC. Several methods for quantitative analysis using GC instruments are available; the USP method uses an internal standard. In GC analysis, the quantity of the injection is very small, and even very minute changes in the volume can cause large errors. The internal standard method makes it possible to correct for these errors.

Obtain the following materials:

1. USP Cholesteryl Caprylate Reference Standard (Cholesteryl n-Octanoate) for the internal standard.
2. USP Testosterone Cypionate Reference Standard for the standard preparation. Follow the procedure for drying as described by the USP.
3. A sample of commercial testosterone cypionate injection. Proceed as directed on page 1327 of USP XXII. The injection sample is a solution in oil, so in order to sample correctly, use a 1.0 mL TC pipette (a TC pipette is To Contain), and rinse the pipette five times with about 10 mL portions of the methanol-water mixture, collecting rinsings in the centrifuge tube. Add 20 mL of methane-water mixture and continue with the USP procedure. (Unless this procedure is followed, the high viscosity of the sample will cause sampling error.) The size aliquot needed depends upon the dosage of injection. For a dosage of 200 mg/mL, use 1 mL.Make duplicate analyses. Make three injections of the standard solution, discarding the first injection. Make two injections of each of the standard and sample solutions. Average the ratios of the standard and sample solutions for use in the calculation.

CALCULATION OF ASSAY CONTENT

(Cs) X (3 mL/3 mL) X (Ru/Rs) X (200 mL/DW) X 100 = % testosterone

where Cs = concentration of standard testosterone, mg/10 mL
Ru = area of the sample under the peak
Rs = area of the standard under the peak

DW = declared weight of drug per unit

NOTE--The above equation is based on a dosage of 200 mg/mL of injection. The declared weight and the sample aliquot must be changed, if the dosage weight of the drug is different.

ERRORS IN GAS CHROMATOGRAPHY

When the external standard technique is used, GC measurements are subject to errors caused by differences in the injected volumes of sample and standard solutions. Each injection is in the order of a few microliters. Any small change in the injection volume causes a large change in response. The internal standard method reduces the errors involved by using a ratio of the response of the internal standard to that of the drug being analyzed, in both the sample solution and the standard solution. When ratios are used, the value obtained does not depend upon the amount injected. Calibration curves can be generated by using a series of dilutions of a stock standard solution; each diluted solution contains the same concentration of the internal standard. If the external standard method of calibration is used, a reproducible injection volume must be made for each sample and standard solution. Much more accurate results can be obtained when the internal standard method is used.

The best GC results are obtained when the retention curves are symmetrical and involve little or no tailing. Adsorption of any compounds on the support causes tailing. If the tailing is large, a column with different polarity may produce a more symmetrical peak. Adsorption usually occurs when the compounds are polar and the support is polar. Decreasing the polarity of the support will reduce the adsorption and tailing. If the compounds are polar, the column polarity must be made more nonpolar to reduce tailing. The chemistry rule of thumb, "Like Likes Like", applies in column selection.

Another cause of tailing is a leak at the septum when air is introduced into the gas flow stream. You should replace the septum when you notice a normally
symmetrical peak of a known compound becoming unsymmetrical. Changes in retention times may also be caused by a leaky septum. Gas flow in GC must remain constant during the entire analysis. If flow is erratic, the retention times will vary. Sometimes the base line will show noise and spikes and will deviate from linearity. A base line that shows such signs usually arises from

a dirty flame detector. Check with your service department about cleaning the flame detector if you are not familiar with the operation. After the flame detector is clean, the base line will usually return to normal.

ADDENDUM A

A. Relative Standard Deviation

The relative standard deviation (coefficient of variation) expressed as a percentage is:

$$S_r(\%) = (100/\bar{X})\left[\sum_{i=1}^{N}(X_i - \bar{X})^2/(N-1)\right]^{0.5}$$

where Xi is the area of the sample peak i and X is the average of the areas of peaks i through N in a series of N injections of the same solution. Refer to USP section <621> on Chromatography for discussion of calculations.

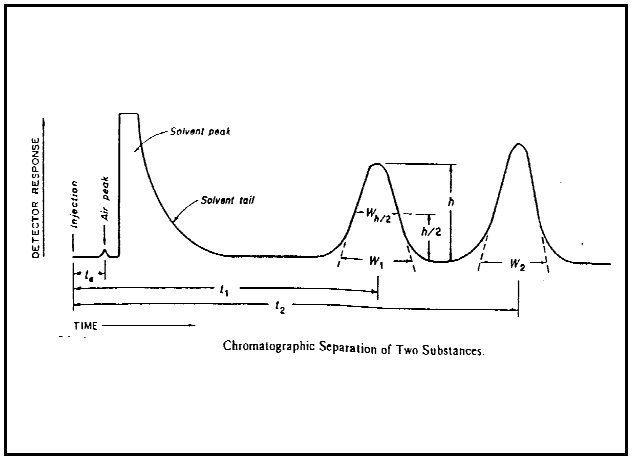
B. Resolution

Resolution is a measure of the separation of two peaks. It is calculated from the widths and the retention times of the two peaks:

$$R = 2(t2 - t1)/(W1 + W2)$$

where W1 and W2 are the peak widths, t1 and t2 are the peak retention times, and W and t have the same units. If the chromatographic parameters are proper, the resolution should not be less than 3.0 for testosterone. Figure 1 shows how the resolution is determined from the chromatogram. This illustration, however will give a resolution of 2 which is less than specified by the USP.

Figure 1



Chromatographic Separation of Two Substances.

Chromatographic Separation of Two Substances

SECTION V

THIN-LAYER CHROMATOGRAPHY TRAINING MODULE

INDEX

THIN-LAYER CHROMATOGRAPHY TRAINING MODULE

Thin-layer chromatography (TLC) is one of the oldest of the liquid chromatography methods. During the past years the method has been largely superseded for drug assay by HPLC methods. Recent developments in coatings have improved TLC by introducing reverse- and normal-phase separations that use the same type of coatings as in HPLC columns. With these new advances, known as HPTLC (high performance thin-layer chromatography), TLC separations can be made comparable to those with HPLC. In fact, the method can be considered as a complement to HPLC, since information gained from TLC can be transferred directly to HPLC. Coatings are available for work in organic or aqueous mobile phases.

TLC is widely used to identify impurities in drugs. The method serves as a valuable means of quick and economical identification. TLC can be used successfully as a rapid screening technique for many drugs. One of its chief uses is determination of trace impurities in drugs. The technique will become even more useful with the new advances in high-performance thin-layer chromatography.

Training Course

USP XXII, section ë621ï, page 1559, provides a background for thin-layer chromatography analysis. The FDA has materials which will help you learn and review the essentials of TLC. Even if you have had prior experience with this technique, you may find it useful to spend some time with the written, audio, and visual instructional materials. You can tailor your instruction to your personal needs. This course will take approximately 1 hour to view and study. You should spend as much time as necessary.

SAVANT Tape-Slide Course, "Principles and Practices of TLC"

- Part 1
  - History
  - Basic components
  - Mechanism of separation
  - Preparation of thin-layer media
  - Selection of mobile phase
- Part 2
  - Variables
  - Basic steps

Causes of poor separation
Applications

INTRODUCTION

The TLC operation requires handling of organic solvents, glass containers, and thin-glass plates. Careful planning is required to make the operation successful and safe. Safety glasses and laboratory coats are required for all operations of this type. The entire process involves the handling of volatile and flammable solvents. All work involving the solvents should be performed in a hood. The thin-glass chromatographic plates are fragile and are easily broken. THE GLASS PLATES MUST BE HANDLED WITH CARE to prevent breakage and possible personal injury. The coated layers used on the plates are also fragile; they should be touched as little as possible to avoid damaging them or affecting development or visualization. The glass plates should be used in a clean laboratory area with space enough to work with small objects safely and efficiently.

The plates require development to separate the materials. The visualization of the plates requires some method of detecting the spots. The most common forms of detection are examining the plate under ultraviolet light, spraying, or charring. All spraying operations must be performed in a spray chamber and in a hood. The charring operation uses H2SO4 with/without a chromate and heating. Rubber or plastic gloves should be worn to prevent any possible injury from burns. Intensities of spots are estimated visually or with the use of a densitometer.

Because TLC operation uses micro quantities of solution, very small micropipettes are used. For preparation of the plates, including application of the sample, the operator should be seated at a table with plenty of clear working space.

PERFORMING A THIN-LAYER CHROMATOGRAPHY SEPARATION

THIN-LAYER CHROMATOGRAPHY EQUIPMENT

Glass tank with a glass lid. The top edge of the tank should be ground so that the tank and lid fit together closely to prevent evaporation. Most operations are carried out in a saturated atmosphere.

2. Saturation pads to provide a controlled atmosphere.

3. Delivery system to handle microliter quantities of sample.

4. Coated chromatographic plates.

5. Spotting template to control sample placement.

6. Spray box.

7. Spray unit.

8. UV light box or UV light source (254 and 360 nm).

All TLC operations include three phases, namely:

1. Stationary phase, which is the adsorbent;
2. Mobile phase, which is the solvent;
3. Solute phase, which is the sample.

Any TLC procedure includes six steps, namely:

1. Sample preparation;
2. Sample application;
3. TLC chamber conditioning;
4. TLC plate development;
5. Visualization, interpretation, and quantitation.

TLC OPERATING PROCEDURE

PLATE SELECTION

TLC plates are available both uncoated and coated. Since you can buy the coated plates with many different types of coatings, it is not necessary to coat plates unless some special type of coating is desired. We will involve ourselves only with the coated plates. The plates themselves are available with glass, aluminum, paper, and polyester backings and also in a wide variety of sizes. The most common size and type used in drug analysis is the 20 X 20 cm glass plate. Most TLC measurements are done with silica coating on glass plates.

In addition to the silica-coated plates, other coatings are available such as C18, C8, CCN, and CPhenyl. These coatings are similar to those used as supports in HPLC. They contain binding agents so that the plates may be used with either organic- or water-containing mobile phases. Special plates are commercially available that will permit the use of up to 50% water. If you attempt to go beyond 50% water, the coating will be removed. The wide variety of coatings makes it possible to separate components by either normal or reverse-phase methods.

Plates come with a soft or hard coating. The soft coatings are used when recovery of the sample is necessary. The hard coatings are abrasion-resistant and will allow marking with a soft lead pencil. Plates are available with or without fluorescein in the coating. Plates with fluorescein allow easy detection of the developed spots under UV lighting. The type of coating on the plate is specified by the USP; usually it is silica.

PREPARATION OF THE TANK

1. Use a clean and dry chromatography tank to fit the size of the plate. Tanks grooved on the inside to hold multiple plates are not recommended.
2. Fill the tank with solvent to such a level that the bottom of the plate dips into the liquid 1 cm. (The tanks used for the 20 X 20 cm plates require about 100 mL of solvent to bring the liquid level to the proper position on the plate.) Make sure the origin line of spotted samples is above the developing solvent in

the tank.

3. Place saturation pads around the inside of the tank, cover with the glass lid, and allow to equilibrate. (Some USP methods specify no saturation pads, stated as "no equilibrium". No mention of saturation pads by the USP means the system is operated with pads.) Allow sufficient time for the developing tank to equilibrate before adding the sample plates.

PREPARATION OF PLATE

1. Use a dry and clean plate.

2. Avoid scoring or drawing lines on the plate that will be traversed by the developing solvent. Mark only the top of the plate to identify the end of the developing time or distance.

3. On the end of the plate opposite the spotting end, mark the edges of the surface to show the upper distance that the developing solvent must travel from the origin (usually about 15 cm). Score the plate between these marks with a sharp object.

4. Do not score along the spotting line.

SAMPLE PREPARATION AND SPOTTING OF THE PLATE

1. Accurately weigh the sample to make a desired concentration (concentrations are specified by the USP). Before application to the plate, dissolve the sample in a solvent that is as nonpolar and volatile as possible.

2. Use a microsyringe or a Microcap to deliver a fixed volume to the plate. A Microcap is a precision-bore capillary tube cut to a constant length so as to deliver a fixed volume in microliters when filled. Microcaps range in capacity from 0.5 to 200 mL. They are disposable and a new one is used for each sample, thus eliminating any possible contamination. Drummond Wiretrol micropipettes with different volumes are calibrated with volume marks and are used to deliver the sample. This type of micropipette consists of a calibrated capillary with a removable wire plunger. Such a system combines the advantages of a disposable pipette and a microsyringe. This type of pipette must be used when the solutions are water or water mixtures because of surface tension. A separate capillary is used with each sample to avoid contamination of samples.

3. With the use of a template or guide, spot the sample slowly, using a microliter pipette along the origin line sufficiently above the bottom of the plate (approximately 2.5 cm) so it is not immersed in the developing solvent when placed in the tank.

4. Spot measured volumes of sample on the origin line across the bottom of the plate. Select a standard and sample of equal or nearly equal concentrations. Spot this standard and sample near the center of the plate on the origin line, and place sample spots of varying concentrations on the sides of the two center spots. Make the sample spots on the plate as small as possible. If all of the sample is added at once, such as the 10 mL specified in most USP analyses, a very large spot of sample will result. To avoid the large initial spot, first add a very small amount of the sample to the plate and allow that portion to dry, using a gentle stream of nitrogen gas to aid in drying the spot as it is applied. The small amount added will dry quickly since the solvent is usually volatile. Continue adding successive small amounts until the correct quantity of sample has been added. Try to keep the spot to about 2 mm diameter for best results.

4. Leave the plate in place and let it dry until you can no longer smell any solvent.

DEVELOPING THE PLATE

1. Place the plate vertically with the bottom down in the solvent tank so that the liquid comes to about 1 cm on the plate.
2. Cover the tank with the lid and allow the sample to migrate up the plate. The USP specifies that the migrate should continue until a certain percentage of the total plate height is reached. If you forget to stop the migration at the time specified, it will stop automatically when the solvent front reaches the scribed line. The usual time for migration is on the order of 30 minutes to 1 hour. Some TLC systems require much longer times to develop; to reduce the time, you must change the polarity of the solvent.

3. If the solvent does not reach the prescored line, mark a line showing where the solvent did migrate.

4. The line where the solvent stops marks the retention distance for the solvent and is known as the solvent front. Use the distance from the origin line to the solvent front to calculate the retention factor (Rf) as described later.to calculate the retention factor (Rf).

VISUALIZATION OR DETECTION

1. Remove the plate from the tank and allow to air dry. If the coating of the plate contains fluorescein, observe the chromatogram by exposing the plate to a short-wavelength ultraviolet light source (254 nm). Place the plate with the coating surface facing towards the UV light source.

2. Alternatively, visualize the chromatogram by spraying the plate with various materials that dye the samples. The type of spray used depends upon the molecular structure of the solute. Use a small atomizer sprayer which eliminates formation of droplets and forms a uniform mist; use a spray coating box to isolate the excess spray. Identify the location of the sample. The intensity of the color developed is related to the amount present.
3. As a third method, use charring to visualize the chromatogram. Spray a mixture of 50% H2SO4/methanol, with/without potassium dichromate, onto the plate and heat to 105EC for a few minutes. The spots containing the samples will be blackened and the intensity can be measured by a densitometer.

4. Measure the distance from the initial sample location (origin) to the center of the eluted spot. Measure the distance from the origin to the solvent front. Express the Retardation Factor, Rf, as a ratio:

Rf = Distance from origin to center of spot/Distance from origin to solvent front.

The Relative Retention Factor, Rr, is defined as the distance traveled by the test substance divided by the distance traveled by the standard.

Rf is a measure of the separation and movement in a given TLC system, and Rr is used to determine the separating power of the system related to decomposition products or related substances. Use a TLC template as a spotting guide and a means of measuring spot size, Rf values, and sample quantities.

ANALYSIS OF SULINDAC

USP XXII, page 1304, describes the analysis of Sulindac. Consult this before starting the analysis.

Obtain USP Sulindac Reference Standard and a sample of Test Sulindac bulk material.

Reference Solution A. Prepare a solution of USP Sulindac Reference Standard in a mixture of chloroform and methanol (1:1) at a concentration of 25 mg/mL. Label this Solution A.

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Reference Solution B. Dilute 1 mL of solution A to 250 mL with the above solvent. Label this Solution B.

Test preparation. Prepare a solution of Sulindac sample with the 1:1 chloroform-methanol mixture at a concentration of 25 mg/mL. Proceed as per USP XXII, page 1304, using the test beginning with System Suitability and followed by Procedure.

COMMENTS ON THIN-LAYER CHROMATOGRAPHY

Densitometers are also used to measure spots on TLC plates but this use is not covered in this training module.
Some coatings are bonded to the glass with a crosslinked-polymeric binder,
making the coating useful with either organic or water/organic solvents in which the water content does not exceed 50%.
Many analyses require development of an HPLC method. TLC offers an efficient and cheap way to develop a method involving the same type of column and solvent system. Data from TLC and HPLC are complementary, and can be directly transferred from one system to the other.

High-performance thin-layer plates can be used to assay the main component in a drug if the analyst can apply constant volumes every time and maintain high precision. Automatic spotters are available to deliver a constant volume and to eliminate operator errors. In some cases, the HPTLC method can be used for the USP method if the HPLC instrumentation is not available.

TlC has the following advantages:

1. Low cost compared to other methods.

2. Ability to screen a large number of samples.

3. No sample clean-up or filtering.

SOURCES OF ERROR

1. Reproducibility of sample application. Very small quantities are handled and any error in the volume applied greatly increases the final error.

2. When using a densitometer, reproducibility of positioning the spot in the center of the beam is a problem. It is very important to center the spot as you scan across, so that your readings are correct.

3. Handling of micro quantities causes some problems because all weighings and volumes are very small and any small change

represents a large percentage change.

4. The sample can over- or under-migrate. Even no separation is possible. All these errors are caused by the solvent polarity not being correct. Sometimes a second solvent front is formed, causing a problem in determining the distance of migration or Rf values.

5. Solvents used in preparing the sample or in the developing solvent can cause decomposition of the sample, giving erroneous results. Solvents of this nature should be avoided.

SECTION VI

ULTRAVIOLET/VISIBLE SPECTROSCOPY

UV/VISIBLE TRAINING MODULE

Analytical methods which are based on the absorption of visible or ultraviolet radiation are frequently included among the official methods used for drug analysis by the FDA. These methods usually require that the analyte be dissolved, chemically transformed to a species which absorbs light, and diluted to a concentration range in which absorbance measurements can produce accurate quantitative results. Training in these methods will consist of learning or reviewing the principles of UV/visible spectrophotometry and the corresponding instrumentation, and a practice analysis on a typical drug sample under the guidance of an experienced chemist, using the USP method.

Training Courses

The FDA laboratory has available training materials which can help you to learn or review the essentials of quantitative UV/visible spectrophotometry. Even if your previous education and experience include some background in these techniques, you may find it profitable to spend some time with written, audio, and visual instructional materials. Below are summarized the contents of two tape-slide courses which are available for this purpose. You can tailor your instruction to your own needs by viewing only those courses or portions thereof which are useful for you. Each presentation takes approximately 1 hour to view. You should spend as much time as necessary with each course, until you thoroughly understand the principles of the technique.

SAVANT Tape-Slide Course UV-101, "Introduction to UV-Visible Spectrometry"

- The spectrum
- Wave-particle duality
- Wavelength units
- The UV-visible range of the spectrum
- Absorption and transmittance of radiation
- Energies corresponding to rotation, vibration, and electronic transitions
- Bonding, non-bonding, antibonding orbitals
- Nomenclature of electronic transitions
- Common UV-visible chromophores (carboxylic acids, ketones, etc.)
- Effect of conjugation on spectra

Interactions between chromophores
Solvent dependence of spectrum
pH dependence
Definition of transmittance and absorbance
Introduction to instrumentation
  Sources (lamp types)
  Monochromator
  Detector (PMT)
  Readout device

SAVANT Tape-Slide Course UV-2, "Basic Aspects of UV/Visible Spectrophotometry
- Instrumentation"

Overview of measurement (single-beam instrumentation)
Sources (tungsten, hydrogen, deuterium lamps, xenon arc)
Detectors (PMT, PbS in near IR)
Electrical signal processing
Monochromator (dispersing element, mirrors, slits)
Angular and linear dispersion
Prisms
Gratings
Single beam (Beckman DU) instrument description
Double beam (Beckman ACTA CIII) description
Effect of wavelength error
Photometric error
Stray light problems
Operating controls of typical spectrometer
Wavelength standards
Absorbance linearity standards

DIODE ARRAY SPECTROPHOTOMETERS

An important type of instrument is not discussed in the SAVANT tape-slide courses, nor is it often covered in college analytical chemistry courses. Unfortunately, the type of instrument that is omitted is also commonly employed in FDA laboratories. These instruments are similar to conventional spectrophotometers except that their detectors are "diode arrays" rather than photomultipliers. A diode array is a strip of sensitive, solid-state photodiodes fabricated in a compact package. Typically, 512, 1024, 2048, or even more diodes are mounted in a package only a few centimeters in length. The intensity of the light impinging on these detectors is determined by a (repetitive) high-speed electronic query of the array. With such a detector mounted at the position which would have been occupied by the exit slit of a conventional monochromator (optics are specially designed to focus on the array, instead of on an exit slit), one obtains virtually instantaneous acquisition of the entire spectrum impinging on the detector. (This characteristic makes diode array spectrometers a desirable, but expensive, detector for HPLC.) Therefore, it is not necessary to "scan" through the spectrum in the conventional sense, and there is only one slit to control, rather than two. Although such an instrument typically does not deliver as high an ultimate resolution as the best conventional monochromators can, its resolution is more than adequate for chromophores in solution. Although neither its dynamic range (the intensity range over which response is nearly linear with photon flux) nor its sensitivity is as large as that of a photomultiplier, both are sufficient for quantitative spectrophotometry of solutions.

If you will be using a diode array instrument and if, like most chemists, you have not had experience with a diode array UV/visible spectrophotometer, it would probably be worth your time to read a brief laboratory guide for the instrument you will be using. An experienced chemist in the laboratory may also be able to help you to become familiar with the instrument.

PRACTICE ANALYSIS

Before beginning the laboratory exercises below, you should have developed the volumetric and gravimetric skills typically required in the preparation of the sample for spectrophotometric measurement. If you are not confident that your skills in these techniques are adequate, you should stop at this point and review or learn afresh those procedures, because you cannot expect to obtain accurate results from any instrumental method unless the quantitative techniques used for sample preparation are of commensurate quality.

A USP method which is representative of UV/visible analysis is the assay of chlorzoxazone tablets (USP XXII, page 303). The purpose of the procedure is to determine the amount of active ingredient in chlorzoxazone tablets. You should perform the analysis under the supervision of an experienced chemist.

PROCEDURE

All of your work must be recorded on a standard worksheet, just as if this analysis were not merely a practice exercise. Every two months, a list of current USP reference standards is published in the Pharmacopeial Forum. The most recent list may be consulted to determine the appropriate standard for chlorzoxazone. The standard (USP Chlorzoxazone Reference Standard) is dried for 2 hours at 105EC. (In order to conserve USP standard materials, you may be directed to use a secondary standard for this analysis. A secondary standard is a material whose purity has been determined by previous analysis.)

ASSAY PREPARATION

Accurately weigh 20 tablets and calculate the average tablet weight (ATW), grind the tablets to a fine powder, and pass the powder through a 60-mesh sieve. Mix the powder thoroughly, and then take a sample portion.

Transfer duplicate weighed amounts, each of which should contain approximately 100 mg of chlorzoxazone, to separate 100 mL volumetric flasks. (Calculate the amount to be weighed from the average tablet weight and the labeled amount in each tablet.)

Add about 80 mL of warm methanol and sonicate for 30 minutes with occasional shaking. Let the solution cool to room temperature, and then dilute with methanol to volume and mix.

Filter through a dry Whatman No. 2 filter (about 10 cm diameter) to a dry flask so as not to dilute the sample. Discard the first 20 mL.

Pipette 2.0 mL of the filtrate into a l00 mL volumetric flask, dilute with methanol to volume, and mix thoroughly.

REFERENCE STANDARD PREPARATION

Dissolve 20 mg of the USP Chlorzoxazone Reference Standard (or the secondary standard) accurately weighed in methanol into a 100 mL volumetric flask and dilute stepwise to obtain a final solution of 20 mg per mL (for example, by diluting a 10.0 mL aliquot to 100.0 mL). Keep in mind that solutions must be clear (no emulsions or precipitates) and spectrophotometer cells must be clean inside and out, if accurate photometric results are to be obtained. Because oils from fingerprints absorb in the ultraviolet, clean the windows of the cell carefully with a tissue made for such purposes, and do not touch them afterward. If the baseline obtained from the blank is rough or jagged, check the outsides of the cells for fingerprints or moisture. Clean the cell and repeat the blank.

Before making measurements on the samples, check the absorbances of the two cells, using the solvent in both cells. Make a correction if the cells are not properly matched (perfectly matched cells do not require correction). Rinse out the solvent and replace it with the solution of the lowest concentration. Rinse the cell at least three times before going to the next higher concentration. Repeat until the highest concentration is reached.

Use the UV/visible spectrophotometer to determine the absorbances of both the sample and standard solutions in the same 1 cm cell, at the absorbance maximum which occurs near 282 nm, using methanol for the blank. Average the sample readings.

CALCULATIONS

Calculate the number of milligrams of the active ingredient in the sample you weighed for this analysis. Use the back of the worksheet to record all of your calculations along with the

analytical data. Average the results from the two runs. Convert the transmittance data to absorbance units using the attached table. If the absorbance is measured on the instrument, no conversion will be necessary.

Determine the percentage of chlorzoxazone in the tablet compared to the declared amount as follows:

(Ws/100 mL) X (10 mL/100 mL) X (100 mL/2 mL) X (Au/As) X (100 mL/Wu) X (ATW/DW) X 100 = % of declared Chlorzoxazone)

where: Au = absorbance of the sample
As = absorbance of the standard
Wu = weight of sample, mg
Ws = weight of standard, mg
ATW = average tablet weight, mg
DW = declared weight of drug per tablet, mg

The allowable percentage of the labeled amount for chlorzoxazone is 90.0-110.0%.

Table 1

CONVERSION OF TRANSMITTANCE TO ABSORBANCE

PercentT	.0	.1	.2	.3	.4	.5	.6	.7	.8	.9
10.0	1.000	.996	.991	.987	.983	.979	.975	.971	.967	.963
11.0	.959	.955	.951	.947	.943	.939	.936	.932	.928	.924
12.0	.921	.917	.914	.910	.907	.903	.900	.896	.893	.889
13.0	.886	.883	.879	.876	.873	.870	.866	.863	.860	.857
14.0	.854	.851	.848	.845	.842	.839	.836	.833	.830	.827
15.0	.824	.821	.818	.815	.812	.810	.807	.804	.801	.799
16.0	.796	.793	.790	.788	.785	.782	.780	.777	.775	.772
17.0	.770	.767	.764	.762	.760	.757	.754	.752	.750	.747
18.0	.745	.742	.740	.738	.735	.733	.730	.728	.726	.724
19.0	.721	.719	.717	.714	.712	.710	.708	.706	.703	.701
20.0	.699	.697	.695	.692	.690	.688	.686	.684	.682	.680
21.0	.678	.676	.674	.672	.670	.668	.666	.664	.662	.660
22.0	.658	.656	.654	.652	.650	.648	.646	.644	.642	.640
23.0	.638	.636	.634	.633	.631	.629	.627	.625	.623	.622
24.0	.620	.618	.616	.614	.613	.611	.609	.607	.606	.604
25.0	.602	.600	.599	.597	.595	.594	.592	.590	.588	.587
26.0	.585	.583	.582	.580	.578	.577	.575	.574	.572	.570
27.0	.569	.567	.565	.564	.562	.561	.559	.558	.556	.554
28.0	.553	.551	.550	.548	.547	.545	.544	.542	.541	.539
29.0	.538	.536	.535	.533	.532	.530	.529	.527	.526	.524
30.0	.523	.521	.520	.519	.517	.516	.514	.513	.511	.510
31.0	.509	.507	.506	.504	.503	.502	.500	.499	.498	.496
32.0	.495	.494	.492	.491	.490	.488	.487	.486	.484	.483
33.0	.482	.480	.479	.478	.476	.475	.474	.472	.471	.470
34.0	.468	.467	.466	.465	.463	.462	.461	.460	.458	.457
35.0	.456	.455	.454	.452	.451	.450	.449	.447	.446	.445
36.0	.444	.442	.441	.440	.439	.438	.436	.435	.434	.433
37.0	.432	.431	.430	.428	.427	.426	.425	.424	.422	.421
38.0	.420	.419	.418	.417	.416	.414	.413	.412	.411	.410
39.0	.409	.408	.407	.406	.404	.403	.402	.401	.400	.399
40.0	.398	.397	.396	.395	.394	.392	.391	.390	.389	.388
41.0	.387	.386	.385	.384	.383	.382	.381	.380	.379	.378
42.0	.377	.376	.375	.374	.373	.372	.371	.370	.369	.368
43.0	.367	.366	.364	.363	.362	.362	.360	.359	.358	.357
44.0	.356	.356	.355	.354	.353	.352	.351	.350	.349	.348
45.0	.347	.346	.345	.344	.343	.342	.341	.340	.339	.338
46.0	.337	.336	.335	.334	.333	.332	.332	.331	.330	.329
47.0	.328	.327	.326	.325	.324	.323	.322	.322	.321	.320
48.0	.319	.318	.317	.316	.315	.314	.313	.312	.323	.311
49.0	.310	.309	.308	.307	.306	.305	.304	.304	.303	.302

Table 1 con't
CONVERSION OF TRANSMITTANCE TO ABSORBANCE

PercentT	0	.1	.2	.3	.4	.5	.6	.7	.8	.9
50.0	.301	.300	.299	.298	.298	.297	.296	.295	.294	.293
51.0	.292	.292	.291	.290	.289	.288	.287	.286	.286	.285
52.0	.284	.283	.282	.282	.281	.280	.279	.278	.277	.276
53.0	.276	.275	.274	.273	.272	.272	.271	.270	.269	.268
54.0	.268	.267	.266	.265	.264	.264	.263	.262	.261	.260
55.0	.260	.259	.258	.257	.256	.256	.255	.254	.253	.253
56.0	.252	.251	.250	.250	.249	.248	.247	.246	.246	.245
57.0	.244	.243	.243	.242	.241	.240	.240	.239	.238	.237
58.0	.237	.236	.235	.234	.234	.233	.232	.231	.231	.230
59.0	.229	.228	.228	.227	.226	.226	.225	.224	.223	.223
60.0	.222	.221	.220	.220	.219	.218	.218	.217	.216	.215
61.0	.215	.214	.213	.212	.212	.211	.210	.210	.209	.208
62.0	.208	.207	.206	.206	.205	.204	.203	.203	.202	.201
63.0	.201	.200	.199	.199	.198	.197	.196	.196	.195	.194
64.0	.194	.193	.192	.192	.191	.190	.190	.189	.188	.188
65.0	.187	.186	.186	.185	.184	.184	.183	.182	.182	.181
66.0	.180	.180	.179	.178	.178	.177	.176	.176	.175	.175
67.0	.174	.173	.173	.172	.171	.171	.170	.169	.169	.168
68.0	.168	.167	.166	.166	.165	.164	.164	.163	.162	.162
69.0	.161	.161	.160	.159	.159	.158	.157	.157	.156	.156
70.0	.155	.154	.154	.153	.152	.152	.151	.151	.150	.149
71.0	.149	.148	.148	.147	.146	.146	.145	.144	.144	.143
72.0	.143	.142	.142	.141	.140	.140	.139	.138	.138	.137
73.0	.137	.136	.136	.135	.134	.134	.133	.132	.132	.131
74.0	.131	.130	.130	.129	.128	.128	.127	.127	.126	.126
75.0	.125	.124	.124	.123	.123	.122	.122	.121	.120	.120
76.0	.119	.119	.118	.118	.117	.116	.116	.115	.115	.114
77.0	.114	.113	.112	.112	.111	.111	.110	.110	.109	.108
78.0	.108	.107	.107	.106	.106	.105	.105	.104	.104	.103
79.0	.102	.102	.101	.101	.100	.100	.099	.098	.098	.097
80.0	.097	.096	.096	.095	.095	.094	.094	.093	.093	.092
81.0	.092	.091	.090	.090	.089	.089	.088	.088	.087	.087
82.0	.086	.086	.085	.085	.084	.084	.083	.082	.082	.081
83.0	.081	.080	.080	.079	.079	.078	.078	.077	.077	.076
84.0	.076	.075	.075	.074	.074	.073	.073	.072	.072	.071
85.0	.071	.070	.070	.069	.069	.068	.068	.067	.067	.066
86.0	.066	.065	.064	.064	.063	.063	.062	.062	.061	.061
87.0	.060	.060	.060	.059	.059	.058	.058	.057	.056	.056
88.0	.055	.055	.054	.054	.053	.053	.053	.052	.052	.051

89.0	.051	.050	.050	.049	.049	.048	.048	.047	.047	.046
90.0	.046	.045	.045	.044	.044	.043	.043	.043	.043	.042
91.0	.041	.040	.040	.039	.038	.038	.037	.037	.036	.036
92.0	.036	.035	.035	.035	.034	.033	.033	.033	.032	.032
93.0	.031	.031	.030	.030	.029	.029	.028	.028	.027	.027
94.0	.026	.026	.025	.025	.024	.024	.024	.023	.023	.022
95.0	.022	.022	.021	.021	.020	.020	.019	.019	.018	.018
96.0	.018	.017	.016	.016	.015	.015	.014	.014	.014	.013
97.0	.013	.012	.012	.012	.011	.011	.010	.010	.009	.009
98.0	.009	.008	.007	.007	.006	.006	.006	.005	.005	.004
99.0	.004	.004	.003	.003	.002	.002	.002	.001	.001	.001

SECTION VII
INFRARED SPECTROSCOPY

INFRARED SPECTROSCOPY TRAINING MODULE

Infrared spectroscopy measures the molecular vibrations of themolecules in such vibrations as bending and stretching. Only those molecules that have some dipole moment or interactionbetween atoms cause absorption in the infrared (IR). The symmetrical molecules such as the stable diatomic molecules (O2,N2, etc.) do not have vibrations in the infrared. When the resonant frequency of the atoms matches the frequency of the energy source, some absorption occurs. The infrared frequencies cover the region of the electromagnetic series from 14000 to 20 cm-1 (wavelength in the IR region is expressed in wave numbers, i.e., the number of waves per centimeter). The IR regionincludes the far and near frequencies. The most importantregion, from 4000 to 400 cm-1, is the standard region, and is the one where most analyses are done. The USP methods use infrared spectroscopy primarily as a means of identifying the drug, but not for quantitatively measuring the strength of the drug in the formulation. In this sense, infrared spectroscopy is a qualitative method in which the absorption spectrum of the analyte is compared with that of a reference standard by matching absorption peaks. However, this training module also includes the quantitative analysis of compounds by infrared spectroscopy.

Instruments using either a salt prism or a diffraction grating to separate the wavelengths require considerable time to measure the entire spectrum. Newer instruments use the Fourier transform method (FTIR) for obtaining the spectrum. This method uses an interferometer which separates all wavelengths in approximately a few seconds, thus permitting a large number of scans. The analyst selects the number of scans needed to produce a satisfactory spectrum. Such a method reduces the noise level.

Many USP monographs specify the infrared spectrum for the identity test of a drug. The infrared spectrum of the sample is measured and compared to that of a USP reference standard material. If the spectra match, the two substances are the same, however isomers such as D- or L- could be present and show as a single compound.

Since infrared spectroscopy is generally not sensitive to trace quantities, low amounts of impurities are not detected. The

method is limited to measuring the main components.

Infrared spectroscopy involving pure drug substances obtained from pharmaceutical drug preparations requires the analyst to handle small quantities of solid materials which have been dried, and are in the form of a fine powder. Such materials are usually subject to static electricity, which makes the finely divided powders jump around when handled. An anti-static gun is useful to prevent this. Most samples are prepared by making pellets of the drug with KBr. The sample and the KBr must be thoroughly mixed. The sample constitutes about 2% of the total mass.

The following general precautions must be observed:
The pellet mold for sample preparation is a precision-fit set of molding dies with close tolerances. Take care to fit the dies together properly; otherwise the mold will be ruined. Clean and dry the molding dies in an oven before making the pellet. Measure both a reference standard and a sample, and try to have the concentrations of the two specimens as close as possible. Use care in operating the hydraulic hand press while making the pellet. Place the mold in the center of the press. Ascertain that the mold and press are properly aligned before applying the pressure. Always apply and release the pressure slowly. Use care in handling the mold to prevent any damage; any scratches on the faces or burring of the edges will ruin the mold. Closely follow all Good Laboratory Practices of weighing and analytical techniques. All infrared techniques use some halide of an alkali metal (e.g., NaCl or KBr) as the sample matrix or sample holder, and the crystals are subject to moisture. Do not handle the faces of the crystals with your bare hands, since any fingerprints or moisture will damage them. When the alkali metal halides pick up moisture, they will become fogged.

The modern FTIR instruments operate with a laser light source which can damage the eye; therefore, do not look into the beam for any reason. The laser power is very low and is safe unless you look directly into the beam. All infrared techniques use some alkyl halide as the sample matrix or sample holder, and these crystals are subject to moisture. When the alkyl halides pick up moisture, the faces become opaque and the transmittance of the light is reduced. Samples and crystals should be stored in a desiccator over silica gel. On high humidity days, it is good practice to sweep the cell compartment with a slow flow of dry nitrogen while making measurements.

Training Courses

The FDA laboratory has available training materials which can help you learn or review the essentials of quantitative infrared spectrophotometry. Even if your previous education and experience include some background in these techniques, you may find it profitable to spend some time with written, audio, and visual instructional materials. Below are summarized thecontents of three tape-slide courses which are available for this purpose. You can tailor your instruction to your own needs by viewing only those courses or portions of them that you need. Each presentation takes approximately 1 hour to view. You should spend as much time as necessary with each course, until you thoroughly understand the principles of the technique.

SAVANT Tape-Slide Course IR 101, "Principles of Infrared Quantitative Analysis"

- History
- Principles of IR quantitative analysis
- Errors in quantitative measurements
- Mathematics of quantitative analysis
- Multicomponent analysis

SAVANT Tape-Slide Course 102, "Techniques of Solid Sample Handling of IR Spectroscopy"

- General spectral criteria
- Solid sample preparations

SAVANT Tape-Slide Course 104, "Computerized Infrared Spectroscopy"

- Digitizing and displaying spectral data
- Housekeeping
- Spectral substitution
- Mixture analysis
- Spectral search

MEASURING THE IR SPECTRA

Sample Preparation

There are several methods for preparing samples for infrared measurement, namely:

1. Thin film of the material. A thin film of the liquid sample (aka - neat technique) is spread between two salt plates. A thinspacer approximately 0.1 mm thick can be inserted to increase absorption, if needed. The absorption is dependent upon the film thickness and any slight variation will cause a change in measured intensity.

2. Material in Solution. The compound is dissolved in a solvent at a very high concentration and then this solution is transferred into a liquid cell. The solvent must have little or no absorption in the region of interest. All solvents will show absorption at some IR wavelengths.

Solvents such as CCl4, CS2, and CHCl3 have absorption bands that allow measurements on most compounds. Suitable analysis can be done when the sample has some absorption band or bands that are different than the solvent. The primary difficulty is that many compounds are not soluble in the nonpolar solvents especially those that are polar which includes most drugs. The method can be used for quantitative analysis when a calibration curve can be constructed based upon Beer's Law.

3. Solids dispersed in a mull. The compound is blended with mineral oil (Nujol) by grinding the material with the oil in an agate mortar. The oil is added slowly until a uniform blend is made with the consistancy of a hand cream. The cream is sandwiched between two salt plates usually without a spacer. This method is subject to some uncertainty due to uniformity of the spread and the thickness. The material is not in solution but in a dispersion which also causes some scattering depending upon the particle size.

4. Solid pellet with KBr. Approximately 2 % of the material is blended with 98 % of KBr by mixing in a wiggle bug to get a uniform mixture. The blend is placed in a Wilmad UNIDIE and pressure applied slowly by a hand hydraulic press until the gage registers 15000 pounds load. The load on the mold is held for 2 min. The pressure is slowly released. A thin transparent pellet is formed. The pellet should be of uniform distribution and the faces should be parallel. There are also several mini-type presses used to press pellets by hand pressure. The pellet method finds the most

applications. The method is especially useful when comparing a sample with a reference standard for identity.

5. Diamond anvil technique. This consists of simply mounting a crystal or small portion of pure material on a diamond mount. The spectrum of the material is recorded where the diamond mount touches the crystal. It is useful for only pure materials and is used with the Fourier transform infrared instruments.

6. Reflectance technique. This technique is used to obtain spectra of difficult to handle material such as paints, fabrics, can liners, etc. Spectra is obtained only of the surface of the sample material in contact with the sample holder wich is usually a thallous bromide-iodide (KRS-5) trapazoidal shaped crystal. The sample material is applied to the crystal to form the best contact possible before the spectrum is attempted. Drug samples are usually evaporated onto the surface from a suitable solvent.

The KRS-5 crystal is held in a special holder which fits into the cell compartment. It is not used much in the drug laboratory.

7. Gas Measurements. Gases are usually introduced into a gas cell which also is specially designed to fit into the sample compartment of the infrared instrument. By adjustment of the mirrors in the cell, path lengths of up to 10 meters can be obtained. This technique is also uncommon in the drug laboratory.

QUANTITATIVE INFRARED SPECTROSCOPY

Infrared Spectroscopy is based upon the amount of absorption of the material and upon the principles of Beer's Law. The measurements are made by transmission which is the ratio of the intensity of the light passing through the sample to the intensity of the initial light which is expressed as T= I/I0 and the percent transmission is the ratio multiplied by 100 which is the manner that the spectum is displayed. (See note). The FTIR instruments display the spectrum in either transmision or absorbance modes.

Beer's law states that log of the recipocal of the transmission is linear with concentration. The law is expressed as:

$$A=abc$$

where A is the absorbance and a and b are constants for any given condition and c is the concentration. The "a" is the absorption coefficent and "b" is the optrical path length. The expression reduces itself to Y=mX + b (the equation for a straight line). This relation holds for most substances over the dilute range but deviates at the higher concentrations. Even with small deviations, the concentration of the analyte can be determined from a calibration curve based upon a second or third order polynominal equation.

Note: Transmittance measurements must be converted to Absorbance before quantitative calculations are made! The attached tables p0rovide a simple means of converting the measured percent transmission to absorbance without making any calculations.

The Absorbance is obtained by the relation $A=\log_{10}(1/T)$ where

A= Absorbance and T= Transmission

USE TRANSMISSION TO CALCULATE ABSORBANCE AND DO NOT USE PERCENT TRANSMISSION. FTIR Infrared Instruments are capable of measuring the transmission or the absorbance as a function of wavelength expressed as wave numbers (cm-1). The wavelengths covered by monochromator instruments depend upon the type grating or the alkyl halide sample matrix or sample holder. Prism instruments scan wavelengths depending upon the dispersion of the alkyl halide prism while diffraction instruments depend upon the grating ruling. Instruments of these types must synchronize the monochrometor with the wavelength markings on the chart. FTIR instruments cover a

wide range from 7800 to 100 cm-1 because all wavelengths are separated and the range is determined by the IR source. The FTIR instruments are controlled by a laser which automatically sets the wavelength and no synchronization is necessary.

Summary of Quantitative Analysis in IR.

Summary of the steps needed to do quantitative analysis by IR.

1. Select analyte absorption band.
2. Choose solvent
3. Get concentration range and pathlength.
4. Measure reference solutions.
5. Construct a calibration curve of absorption versus wave numbers (Not in every case).
6. Determine unknown concentration.

Setting the concentration and the path length should be made so that the transmission of the sample lies between 20 and 80 %. If either the concentration or the path length is too great, some of the stronger bands will bottom out and some absorption bands could be missed. If the transmission is too large, then the response from the sample may not have sufficient absorption to show some of the less intense absorption bands.

LABORATORY EXPERIMENT. Assay and Identity of Meprobamate in Tablets

Analysis of Meprobamate Tablets. Official Methods of Analysis (AOAC) Book of Methods, 14th ed., 1984, p. 699. (modified for the purpose of this training).

Obtain from your supply source a sample of USP Meprobamate Reference Standard and Meprobamate tablets. The Reference Standard should be dried according to the label on the standard bottle. Since tablets contain some excipients as well as the main drug, it is necessary to separate the main drug from the excipients because all components of the tablet will contribute to the IR spectrum. In some cases,the bands from the active ingredient may be in an area free from excipient interference and you can take advantage of this.

PROCEDURE

Preparation of sample solution. Obtain the average tablet weight using 20 tablets(ATW in mg). Reduce the tablets to a fine powder with a mortar and pestle and sieve through a 60 mesh drug sieve. Mix the powder thoroughly. Take a portion of the powder equivalent to 100 mg of Meprobamate and place in a 100 ml volumetric flask. Add 50 ml of anhydrous chloroform(SEE NOTE FOR PREPARATION). Place in an ultrasonic bath for 15 minutes with frequent agitation or shake for 20 minutes. Add anhydrous chloroform to volume and mix. The drug and the excipients are separated on the basis of solubility since most excipients will be insoluble in the chloroform. With a 10 mL hypodermic syringe draw up the solution and then filter a portion through a 25 mm Nylon filter of 0.45 micron porosity discarding the first 1-2 ml. Fill the IR cell with the next portion of filtrate.

NOTE(Preparation of Anhydrous Chloroform)

1. The chloroform is washed using equal volumes of water in a separatory funnel and shaking for one minute. Discard the upper layer. The washed chloroform must be used within 2 days. If not used within the time limit, the chloroform must be rewashed.
2. Filter the washed chloroform through a bed of anhydrous sodium sulfate to obtain the anhydrous chloroform.

Prepare a standard solution of USP Meprobamate of 100 mg/100 ml in anhyd. chloroform.

Before transmission measurements can be made, it is necessary to calibrate all monochromator instruments using a film of polystyrene(normally supplied with the instrument). The polystyrene bands should correspond to the wavelengths on the chart. If the wavelengths do not match, then you must adjust the chart drive to correspond to the monochromator. Calibration is not necessary with FTIR instruments since the wavelengths are set by a laser and the polystyrene spectrum is stored in the instrument memory.

Scan the sample and standard solutions in 1.0 mm sodium chloride matched cells from 5.0 to 6.5 microns (2000 - 1540 cm-1) against anhyd.chloroform as the reference. Make 3 scans of the sample and standard and average the results. Make sure that you select the right cell thickness with a spacing of 1.0 mm. Cells with less spacing will not give enough absorption to show a suitable spectrum. After completing the IR scans, thoroughly rinse the cell with chloroform to remove all traces of the samples. Any remaining compounds will show up in future measurements. Store the cleaned cell in a dessicator.

Calculate meprobamate by subtracting Absorbance at 5.5 microns (1818 cm-1) from Absorbance at 5.82 microns (1718 cm-1) and compare with the standard. Compute the percentage of meprobamate by the following relation:

IDENTIFICATION OF MEPROBAMATE

Filter the remaining solutions of sample and standard to remove any insoluble excipients. Take about 25 ml of assay solution and evaporate to dryness on a steam bath. The evaporation is best carried out as follows:

1. Put the 25 mL of solution in a 100 mL beaker and evaporate to approximately to 5 mL.
2. Transfer the partially evaporated solution to a small testtube and continue evaporation until all visible solvent has disappeared.
3. Dry the residue in a vacuum oven at 60EC for 3 hours. Cool and weigh about 2 mg of the residue and mix with 98 mg of KBr in an agate mortar. Grind until homogeneous. Prepare a pellet of the mixture using either the hand held mini-press or the Carver press. Mount the clear transparent pellet in a holder and record the infrared spectrum. (Note the positioning of the cell so that the standard and sample can be run with the same cell orientation). Compare with a standard prepared similarly.

The infrared spectra should match in every respect.

When you compare the spectra of the standard and the sample, you should have at least as many absorption bands in the sample as in the standard. Identity should never be established on fewer bands. If the bands do not match, then why not. Remember that any other components will contribute to the spectrum. In the case of drug analysis, the cause is usually associated with the presence of excipients.

ERRORS IN INFRARED ANALYSIS

1. Quantification is difficult when the samples are prepared by the mull or pellet methods.
2. Obtaining a homogeneous mixture of the sample either in the mull or pellet.
3. Separating the individual components of a mixture because of complicated spectra and over lapping absorbance bands.
4. Making the pellets with parallel faces without striations.
5. The infrared method is not as sensitive as other methods and can not be used to detect trace amounts. High precision can be obtained but the accuracy may suffer when compared to other methods.
6. Solubility problems especially with the polar drugs.
7. Absorption band used for quantification must be isolated

and free from interference.

8. Measurements made in transmittance mode must be converted to absorbance before calculating results.

9. Liquid cells of matched thickness are needed for qualitative and quantitative results.

10. Excipients in the formulation interfere with the assay or identity.

SECTION VIII

DISSOLUTION

DISSOLUTION TRAINING MODULE

Most drugs in the form of tablets or capsules are required to dissolve in the body within a certain time. Many of the tablets have a coating to achieve a certain time or place of release in the body. Dissolution means the dissolving of the solid drug in the formulation into a specified medium (solvent). Soft capsules may or may not have a dissolution specification because this form of dosage usually contains a liquid.

The container of commercial tablets or capsules bears labeling which indicates the amount of active drug per unit. The result of the dissolution measurement in the USP XXII is expressed as "Q", which is the percentage of the labeled amount of active drug released in the specified time.

Methods have been developed for measuring dissolution by using water or simulated body fluid mixtures. The in-vitro dissolution test correlates closely with the ability of the drug to dissolve in the human system. Some drugs require that solubility be measured as a function of time; others specify a fixed time only.

This module is aimed at assisting the analyst to develop the necessary skills for measuring drug dissolution in a quantitative manner.

The following references form a part of the training and should be studied before the laboratory portion of this module is performed:

1. Cox, D.C., Furman, W.B., Moore, T.W., and Wells, C.E. Guidelines for Dissolution Testing: An addendum, Pharmaceutical Technology, Vol.8, No. 2, February 1984, pages 42-44 (copy attached)*.

2. USP XXII, ë711ï, page 1578, describing DISSOLUTION.

The MOC will have the Hanson dissolution instrument which consists of three basic components, as follows:DDA:

1. Temperature-controlled bath set at 37 % 0.5EC. The water level in the bath should be adjusted to just below the water level in the kettle when either 500 or 900 mL is used inside the kettle.

2. Six covered kettles, each 1000 mL capacity with a hemispherical bottom. The kettles may be either transparent glass or plastic.

3. Variable speed stirrers. All stirrers operate at the same speed and are driven by a belt and gears.

Dissolution can be measured by two different methods: (1) the basket method and (2) the paddle method. The USP designates the basket method as "Apparatus 1" and the paddle method as "Apparatus 2".

INSTRUMENT SETUP

Setting up the instrument and aligning the system is the most important step in dissolution measurements. Follow the procedure carefully.

PRELIMINARIES

Before beginning the instrument setup, obtain the following standards and dry them according to instructions of the USP:

1. USP Aspirin Reference Standard.

2. USP Salicylic Acid Calibrator Standard (non-disintegrating type).

3. USP Prednisone Calibrator Standard (disintegrating type).

Store all standards in a desiccator until the analysis is complete.

I. ALIGNMENT OF DISSOLUTION UNITS

A. Adjust the bottom of the unit until the base is level, as indicated by a bubble level.

B. Level the individual kettles by placing tape underneath each kettle.

1. The Hanson unit, kettles and kettle positions are numbered 1 to 6, starting from the front of the unit, left to right, then the back of the unit, left to right.

2. Glass kettles are not interchangeable. Once leveled, they should be marked and from then on placed in the same hole of the instrument facing the same direction.

C. With a bubble level, make the paddle or basket shafts vertical by using the adjustments on the sides and the back of the Hanson dissolution unit.

D. Center the Hanson units.

1. Center the kettles with the centering tool for the Hanson units.

2. Put water in each kettle to keep it from floating if water is in the dissolution bath. If the shafts are teflon-coated, turn them upside down. Place a centering tool on each shaft; lower it several times on top of the kettle until it centers. Tighten the three retaining rings.

3. Plastic kettles are interchangeable after centering.

4. Glass kettles are not interchangeable. Once centered they should be marked and from then on placed in the same hole of the apparatus, facing the same direction.

E. Adjust the height of each shaft to 25 % 2 mm from the bottom of the paddle or basket to the inside bottom of the kettle, using a depth gauge. On the Hanson unit, set the lower collar on each of the three stainless steel rods 4.5 cm from the base of the unit. On both units, lower the assembly to rest on the collars. Place the depth gauge on the bottom of each kettle, lower the paddle or shaft containing the basket to the top of the gauge, and tighten the shafts. When using baskets, mark their positions by wrapping tape on the top of the shafts .

F. Keep the vibration of dissolution apparatus to a minimum.

1. The external heater/circulator must not touch the dissolution apparatus.

2. The dissolution apparatus motor or other motors nearby must not cause shafts to vibrate.

G. Make sure that the belt drive of the apparatus rotates smoothly; oil, grease, or change bearings if necessary.

H. Fill the dissolution water bath so the level is just below the water level in the kettles when they are filled with 500 or 900 mL.

I. Add Clear Bath (part number 105540, Spectrum Medical Industries, Inc., 60916 Terminal Annex, Los Angeles, CA 90060), 5-6 drops per gallon, to the bath to prevent algae formation.

J. If desired, put the holding tank heater and the heaters used for the Hanson and Distek unit on timers so that the water is preheated when you come in.

II. PREPARATION OF DISSOLUTION MEDIUM FOR RESPECTIVE DRUG

A. Degas all dissolution medium daily, as follows:

Fill a glass carboy with up to 18 liters of dissolution medium (the USP method specifies the medium for each drug), and place the 2-holed stopper with the two pieces of glass tubing into the carboy (the carboy must have some glass tape on the outside to prevent implosion). Turn on the vacuum, allow air to be introduced into the bottom of the carboy, set the pressure to 140-150 mm mercury, and degas for at least 20 minutes with a high velocity vacuum pump (low velocity systems will not work).

B. Treat the degassed medium as follows:

1. Siphon the degassed medium into six 500 or 900 mL volumetric flasks, filling to the mark. Place in a water bath at approximately 38EC until the medium equilibrates (approximately 20 minutes).

2. Slowly pour the contents of each flask of the equilibrated medium down the inside walls of the dissolution kettles. This helps prevent splashing and introduction of air into the kettles.

3. Check the temperature of the dissolution medium in the kettle. The temperature must be 37 % 0.5EC before the test begins.

III. PROCEDURE FOR SAMPLE DISSOLUTION BY THE BASKET METHOD (APPARATUS 1)

A. Place one tablet in each basket and attach to the basket shaft. When the baskets are out of the bath, they are in the up position.

B. Start rotation, check the revolutions per minute (rpm) of the shaft with a stopwatch, and adjust if necessary.

C. Lower basket shaft #1 to the preset depth into kettle #1 and lower each of the next numbered baskets into its kettle at 1 minute intervals thereafter.

D. Place the evaporation cover on each kettle.

E. At the end of the specified dissolution time withdraw an aliquot of dissolution medium from kettle #1. (NOTE: The following steps described in section E are for manual operation.)

1. Using a 50 mL syringe with a glass cannula (a cannula is a small tube for insertion into another vessel), withdraw approximately 50 mL at a point midway between the top of the paddle and the top of the dissolution medium and midway between the shaft and inside wall of the kettle.

2. Take off the cannula and attach a plastic filter holder containing a 0.45 mm filter (the filter must be water-compatible). Discard the first 10 mL of the filtrate and collect the rest.

3. Repeat steps 1 and 2 above at 1 minute intervals for the remaining kettles, using clean syringes and filters for each kettle.

4. Rinse the shafts with deionized water and 95% alcohol, and wipe with a Kimwipe.

5. Empty the kettles and rinse the paddles, kettles, syringes, filter holders, and cannulas with deionized water and 95% alcohol. Place the syringes, filter holders, and cannulas in a drying oven to speed evaporation if desired.

6. Analyze all samples by the method described in the USP. (In automated methods, the sample is automatically analyzed at the end of each dissolution period.)

IV. PROCEDURE FOR SAMPLE DISSOLUTION BY PADDLE METHOD (APPARATUS 2)

A. Place all paddles in the kettles.

B. Place the evaporation covers on the kettles.

C. Start rotation, check the rpm of a shaft with a stop watch, and adjust the speed if necessary to within % 4% of the desired rpm.

D. Drop the first tablet in kettle #1 and drop another tablet in kettles #2, 3, 4, 5, and 6, respectively, at 1 minute intervals thereafter.

E. Perform steps 1-6, section III E.
The use of the USP calibrator tablets do not indicate proper equipment setup for the paddle method as shown by Cox,Furman, Moore, and Wells1.

V. ANALYSIS OF THE DISSOLVED DRUG

The USP specifies the method of analysis, usually by either UV/visible spectroscopy or HPLC. In either case, use aliquots of the collected samples. See the training modules for either UV/visible spectrophotometry or HPLC to obtain operational expertise.

VI. REFERENCES

For additional information on dissolution, see USP XXI, pages 1243-1246,2464-2466, and 3079-3082, and "Guidelines for Dissolution Testing: An Addendum" by Cox et al. (attached).

APPARATUS SUITABILITY TEST

After the dissolution apparatus is set up, it must be calibrated with USP calibrator standards. Use both the USP disintegrating type (Prednisone 50 mg, current lot) and the non-disintegrating type (Salicylic Acid 300 mg, current lot) at 50 rpm with Apparatus 1 (basket). Use Tables 1 and 2 for the preparation and suitability requirements. If the results fall outside the specifications for standard solute (Table 3), check the apparatus and solvent to find the source of the trouble. If the results are acceptable, proceed with the sample of aspirin tablets by the method in USP XXII, pages 113-114.

PREPARATION OF STANDARDS FOR SUITABILITY TEST

The following is a guide for the preparation of standard solutions and the dilutions (if needed) for the analysis of the calibrator tablets. The guide is given for Apparatus 1 and 2 at 50 rpm.

PREDNISONE

Prepare a 25 mg/25 mL stock solution of USP Prednisone Reference Standard in 50% ethanol.

Prepare 12 liters of deionized water and degas as for a dissolution medium.

Make all dilutions with the dissolution medium and measure the UV absorbance at 242 nm. Use the dissolution medium as reference for UV. The percent of prednisone is calculated by the relation:

$$(Cs) \times (Au/As) \times (2\ mL/200\ mL) \times (900\ mL/50\ mg) \times 100 = \%\ Prednisone$$

where: Cs = concentration of standard prednisone, mg/25 mL
Au = absorbance of the sample
As = absorbance of the standard.

SALICYLIC ACID

Prepare 9 liters of pH 7.4 buffer by weighing and mixing 61.25 g of potassium phosphate monobasic and 27.23 g of 50% aqueous NaOH solution. Use a pH meter to adjust to pH 7.4, if necessary. This buffer is the dissolution medium.

Prepare a 25 mg/25 mL stock solution of USP Salicylic Acid Reference Standard in 95% ethanol.

Make all dilutions with the dissolution medium. Measure the UV absorption at 296 nm. Calculate the percentage of salicylic acid by the relation:

(Cs) X (Au/As) X (3 mL/200 mL) X (15 mL/50 mL) X (900 mL/300 mg) X 100 = % Salicylic acid

where: Cs = concentration of standard salicyclic acid, mg/25
Au = absorbance of the sample
As = absorbance of the standard.

If the calibrators show that the apparatus requirements are met, proceed with the aspirin dissolution test. Use the procedure for aspirin tablets described in USP XXII, page 113. Make up 9 liters of an acetate buffer for the dissolution medium by weighing 26.91 g of sodium acetate trihydrate and 14.94 mL of glacial acetic acid and diluting to the required volume with deionized water.

Prepare a standard solution of aspirin by weighing an amount of USP Aspirin Reference Standard approximately equivalent to 100% dissolution (i.e., 325 mg of aspirin in 500 mL of acetate buffer equals 65 mg per 100 mL) into a 100 mL volumetric flask. (NOTE--Do not exceed the 65 mg weight.) You may use some alcohol to dissolve the pure aspirin, but do not exceed 1%, based

upon the final concentration. In this case, add 1 mL of alcohol to the weighed powder, and dilute to volume with the acetate buffer.

Perform all dissolution tests in units of six tablets. When the criteria are met at any stage, do not perform any further testing. If there is failure at any stage, proceed to the next stage of testing until all three stages have been completed. The sample is not considered to fail to meet specifications until all three stages of test have been completed. If one or more tablets fall below Q-25, or if more than two tablets fall below Q-15, stop the test after 12 units. A minimum of six units and a maximum of 24 units are required for any acceptance or rejection of the drug.

Sampling may be done manually or automatically. If automatic sampling is available, the time of sampling must be entered into the program before measuring (see the discussion under Hewlett-Packard Automatic Sampling System). The dissolved sample and the standard are measured by UV at 265 % 2 nm.

The amount dissolved is calculated by the relation:

(Cs) X (Au/As) X (500 mL/325 mg) X 100 = % Aspirin

where Cs = concentration of standard aspirin, mg/100 mL
Au = absorbance of the sample
As = absorbance of the standard.

The acceptance criterion for aspirin is 80% dissolved in 30 minutes.

USP defines the amount dissolved as a percentage of the labeled amount of the active ingredient as "Q". All dissolution measurements have an acceptance criterion as shown in the Table .

ACCEPTANCE CRITERA FOR DISSOLUTION

Stage	No. samples tested	Criteria
S1	6	Each unit not less than (Q + 5%)
S2	12	Average of 12 units (S1+ S2) is equal to or greater than Q, and no unit less than (Q -15%)
S3	24	Average of 24 units (S1+S2+S3) is equal to or greater than Q; not more than 2 units less than Q -15% and no unit less than (Q -25%).

DISSOLUTION BY APPARATUS #2

If time permits, test the calibrators and aspirin sample by the paddle procedure.

Many of the USP dissolution methods specify Apparatus #2, which is the paddle method. The setup of the instrument and the alignment of the kettles and shafts are the same as those specified for the basket method. The difference is in the manner of introducing the sample into the medium and the type of stirrer. All other operations are the same as these described for the basket method.

Drop the tablets down the side of the kettle. Mount each capsule in a helix-shaped wire holders and drop them down the side of the kettle so that the capsule will sink. After aligning and filling the kettles with the medium, lower all stirrers into the medium and start rotation. Set the stirring speed to within % 4% of the designated rate with a stopwatch. Drop the first tablet (or weighed capsule) down the side of kettle #1. After a short delay, e.g., one minute, drop the next tablet into kettle #2 and so on, until all six samples have been added to the respective kettle. Sample each kettle and filter after the dissolution time is reached.

When all six samples have been completed, wash off all stainless steel parts of the apparatus with deionized water and rinse them with 95% alcohol. Dry the parts with a Kimwipe. When using acid dissolution medium, do this immediately after completing the test to prevent corrosion.

SUMMARY OF STEPS IN DISSOLUTION

1. Dry the standards.

2. Set up the instrument and align all components.

3. Prepare the dissolution medium for salicylic acid (phosphate buffer, pH 7.4) for instrument suitability and degas.

4. Prepare a standard solution of salicylic acid in dissolution solution, and perform the dissolution test on the USP dissolution standard (non-disintegrating type). Measure the amount of salicylic acid by UV at 296 nm.

5. Prepare the dissolution medium for prednisone (deionized water) and degas.

6. Prepare a standard solution of USP Prednisone Reference Standard in dissolution medium, and perform the dissolution test on the USP dissolution standard (disintegrating type). Measure the amount of prednisone by UV at 242 nm.

7. Determine the instrument suitability. If instrument standards are not met, determine the cause.

8. Prepare the dissolution solution for aspirin (acetate buffer, pH 4.5) and degas.

9. Prepare the aspirin standard, using 1% alcohol, and perform the dissolution test on aspirin, using Apparatus I (basket). Measure the amount of aspirin by UV at 265 nm.

10. Calculate the results.

11. Determine if the sample meets USP specifications.

12. Repeat all of the above steps, except those for the standard preparations, using the Apparatus II (paddle method).