Rapid Screening of TB Pharmaceuticals

by

Thin-Layer Chromatography

by

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Rapid Screening of TB Pharmaceuticals by Thin-Layer Chromatography

Introduction

Thin-layer chromatography provides a quick, economical, and reliable method for rapid screening of pharmaceuticals. The screening method can be used after little training, and in areas outside the laboratory. This compendium of drug analytical methods has been developed for rapid screening of drugs in such places as ports of entry, pharmacies, distribution centers, or areas lacking resources for other methods of analysis. The technique reduces the need for other analytical methods which are more costly and time consuming, and requiring highly trained operators. The methods are based on a portable system using a plastic bag for development and are easy to use in field-type operations. None of the methods described are official.

In working with any chemical, safety and disposal must be considered before performing an analysis. All chemicals are toxic, and should be handled accordingly. The analyst should not breathe or inhale vapors or dust from any of these chemicals, including the dust from the finely divided silica on the TLC plates. Plastic or rubber gloves should be used whenever contact with these chemicals are possible. An effort has been made to reduce the risk of toxicity of the solvents by using smaller quantities to reduce exposure and by eliminating toxic chlorinated solvents. The toxicity of the chemicals used in these methods is similar to that of solvents used in applying paint. All analyses should be performed in areas with adequate ventilation. The rules of disposal for your local area should be followed. Chemicals used in TLC operations are flammable, and must be kept away from flames or ignition sources. Iodine will stain the skin and clothing, so wear protective clothing and rubber gloves when handling it. Ninhydrin upon contact also stains the skin to a dark spot. Wear rubber gloves when handling ninhydrin in any form.

This compendium describes the procedures for the analysis of the TB drugs in which rapid TLC can be used as a screening method. These methods were developed in our laboratory and have not been collaboratively tested. If problems are encountered with any of these test methods, please notify us by FAX or by mail marked to the attention of the Director, Division of Testing and Applied Analytical Development, Food and Drug Administration.
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Rapid Screening of TB Drugs by Thin-Layer Chromatography

The TB drugs come in many different contents so the drugs described herein represent only a single content; for different contents, the solutions need to be adjusted for proper concentrations needed for suitable detection. The TLC methods described here are semiquantitative. The method gives a good estimate of whether the drug is the same as that listed on the label, and if the content is the correct amount as specified. It is not intended to replace any official compendium method. The drugs selected were those that are currently being used for treatment of TB.

The TLC method was designed to rapidly screen drugs by using a polyethylene bag as the developing chamber. Two reference concentrations representing the upper and lower concentration for the dosage limits (85% and 115 or 120%) are spotted on a plate along with the sample solution representing 100%. The sample solution is spotted between the reference solutions. The spots are examined visually either by ultraviolet light or by iodine staining. The drug is considered to be within specifications if the intensity of the sample spots lies between the intensity of the high and low reference solution spots. The sample should be further tested by an official method if the intensity of the sample spot lies at /or near the lower limit or outside of the lower spot. The screening method eliminates the need for further analysis of those drugs which show concentrations within the specification range.

Drugs other than the TB drugs may need to be analyzed. Methods can be developed for these drugs by following these simple steps:

1. Determine a suitable solvent system by studying the molecular structure of the drug; consult some reference book such as the Merck Index for a suitable solvent. Choose the solvent with the lowest polarity when more than one solvent is possible.
2. Prepare a solution of the standard drug at a concentration approximately equal to 1 mg/mL. Spot this solution on a TLC plate.
3. Prepare a developer solution mixture to have a middle range of polarity, such as equal volumes of toluene and methanol. Add a small amount of glacial acetic acid if the drug is acidic; add concentrated ammonium hydroxide if the drug is basic. Dip the spotted plate into a beaker containing the developer to select the mixture. Cover the beaker to prevent evaporation, and observe the movement of the spots.
4. Reduce the polarity of the developer if the spots follow the solvent front; increase the polarity of the developer if the spots do not move or move less than 1 cm.
5. Adjust the concentrations to show differences in spot intensities after determining the solvent mixture for suitable separation,
6. Add the new method to your list of procedures for later use.

The concentrations specified for the sample and standards were determined experimentally to give suitable detection. These concentrations were determined on the basis of spotting 3 microliters of the solution each time. Drugs may be supplied in dosages other than those listed. The final concentration of the sample should be kept the same as the listed concentration when other dosages are used and may be prepared either by using larger volumes of solvent or by diluting a concentrated solution. Diluting a concentrated solution will use less solvent than preparing the final solution with no further dilution. The drug may also be supplied in different dosage forms, such as liquids. Drugs in liquid form are handled on a volume basis (mg/mL) and

are diluted if necessary.

The availability of reference standards and their cost is a matter of concern to all who analyze drugs. Reference standards have been developed in the form of tablets for the TB drugs. These tablets contain a predetermined quantity of the drug when dissolved in a fixed volume of solvent the correct solution concentration for the high reference solution will be prepared. No weighing is required when reference tablets are available, but at present, the availability of reference tablets is limited. Therefore weighing may be necessary. Also procedures have been written for standards supplied as primary / secondary standards when the reference tablets are not available. Primary standards are costly, but secondary standards can be used successfully. Secondary standards may be obtained from a previously analyzed sample or from reputable chemical suppliers. When either primary / secondary standards are used, the standards must be weighed on an analytical balance capable of at least weighing to 0.1 mg, and a large enough quantity must be weighed to minimize the error. The error can be further reduced by using a semi-micro balance (one that weighs to the 5th place or 0.01 mg).

The TLC procedures described are based on the use of a portable kit supplied with plastic bags, holders, and all the accessories required to perform the analysis. Volumes used in the compendium methods are those suitable for a flat plastic bag 8 cm wide. Some kits have been supplied with plastic bags 10 cm wide which require 30 mL of the developer; therefore all volumes of the developer mixtures must be adjusted by increasing each volume by 50%. The flat 8 cm plastic tubing can be obtained in rolls (006 gage), and bags fabricated from the 8 cm tubing by using a bag sealer. It is recommended that a roll of flat plastic tubing and bag sealer be purchased to insure an adequate supply over an extended period, and to reduce the cost of developer solvents. The developing bags can be reused until a leak in the bag is developed.

TLC plates are available with many different coatings and supports. The methods developed in this compendium are based on plastic-backed silica plates containing a fluorescent material. Merck plastic-backed plates designated as 60 F254 have been found most satisfactory. TLC plates made by other manufacturers are acceptable if they have the same specifications. Coated glass plates are suitable, but will increase the cost. A plate 5 X 10 cm is required for the apparatus. Cutting glass plates from larger plates is not recommended. Aluminum-backed plates have also been satisfactory when used with developers that are not too strongly acidic or basic. Aluminu TLC plates without the fluorescent materials cannot be used for ultraviolet detection; the detection must be done by other means. Keep the plates separate to avoid mistakes when both types are used. Plain silica-coated plates without the fluorescing material are easily damaged and do not have as good of a separation. The 60 F254 plastic- backed plates give the best all-around performance.

The bag for iodine staining can be made as follows: Cut the development bag approximately 12 cm above the seal. Cut a slit in one side of the bag approximately 9 cm above the seal. Place some protective covering on a vertical surface to protect the surface from stain. The protective covering can be cardboard, plastic film, or any other type of material which can be discarded. Tape the bag (top of the bag above the slit) to a vertical surface on top of the protective covering. Tape the bottom of the bag to the vertical surface. Tape a small, flat, rigid object to the bag at the seal point of the bag so that the rigid object can act as a hinge to displace the iodine solution upwards.

The aminoglycosides such as streptomycin and kanamycin must be stained either with iodine or ninhydrin. Staining by the ninhydrin solution requires the use of a bag like the one

described above for the iodine staining.

The TLC analysis is based on the use of one dosage unit to prepare the needed sample solution. Place the complete ground tablet in a small polyethylene bag and grind to a fine powder; add the bag and contents to the vessel and then add the proper volume of solvent to prepare a solution representing the 100% solution. A bag approximately 5 X 8 cm is adequate. A bag of this size can be prepared from the flat 8 cm plastic tubing by sealing the bottom of the bag and then cutting the bag from the tubing. Seal along the side to make a bag width of 5 cm. Cut off the excess width of the bag. Drugs in capsule or powder form do not need to be ground and do not require a bag for grinding.

Other developers produce different heights of the spots and different times for the solvent front to reach the migration limit. Final spot positions should be kept between Rf of 0.2 and 0.8 for best results.

The analysis should be repeated to verify the result when any drug is shown not to meet specifications. The drug should be submitted for analysis by an approved method when the result shows a marginal content near the 85% level. Most analyses will show a drug to be near the midpoint between the upper and lower reference solutions, thus eliminating the need for further analysis.

Preparation of sample solutions

Two methods for the preparation of sample solutions are possible depending on the availability of an analytical balance.

1. Analytical balance available.

The solutions are prepared by using an aliquot method. Weigh the dosage form (such as a tablet or capsule) and determine the fraction of the active drug. For example, a rifampin tablet weighs 400 mg and the declared content is 300 mg, then the fraction of rifampin is 300/400 or 0.75. For capsules weigh the capsule with the contents; empty out the contents and reweigh the capsule alone. Subtract the capsule weight from the total weight to obtain the weight of the contents. Calculate the fractional content of the drug using the declared weight content of the drug.. Grind the tablet to fine powder after the fraction has been determined. Drugs in capsule form do not need to be ground. Weigh an aliquot and calculate the actual weight of drug using the fractional content. Add the proper volume of solvent to make the desired concentration to represent 100%.

For example: The sample contains a drug fraction of 0.75 of the total weight, and you weighed 11.25 mg of the ground powder, multiply 11.25 X 0.75 =8.44 mg(rounded off) to obtain the actual weight of the drug. Then add 8.44 mL of solvent to obtain a required concentration of 1 mg/mL. Measure 8 mL with a pipet and the 0.44 mL with a 1mL tuberculin syringe (graduated).

2. Analytical balance not available.

Add one tablet to a small polyethylene bag and grind to a fine powder. The bag and contents are added to a suitable vessel, the proper volume of solvent added to prepare a concentrated solution which must be further diluted by taking an aliquot for the TLC sample solution. Sample solutions from capsules are prepared by carefully emptying the contents into a suitable vessel and adding. Drugs come in many different contents so that you need to alter your quantities of solvent to prepare the TLC solution. This solution represents the 100% solution and must be accurately prepared.

For example: You need a final solution concentration of 1 mg/mL and the tablet you have contains

200 mg of the drug, grind and dissolve the entire tablet in 20 mL of solvent to make a solution concentration of 10 mg/mL. Take 1 mL of that concentrated solution and add 9mL of solvent to prepare the final solution (1 mg/mL). Make certain that the volume you add to prepare the concentrated solution is sufficient to dissolve the drug.

Preparation of reference solutions:

A. Reference tablets available

The cost and availability of reference materials are a problem in all areas of the world. To make reference materials more available and at lower cost these compounds have been developed in tablet form which requires no weighing. One tablet containing a predetermined quantity of the drug is used to prepare the high reference solution.

Reference tablets for the TB drugs have been prepared and require no weighing for preparation of reference solutions. Add 5 mL of solvent to obtain a solution equivalent to 115 % when the sample is prepared to be 100%. In case of the streptomycin or other antibiotics, the high reference solution will have a concentration equivalent to 120%. Prepare the low reference solution by taking 1mL of the high reference solution and adding 0.35 mL of solvent to make a solution equivalent to 0.85%. Take 1 mL of the high reference solution if the drug is antibiotic and add 0.41 mL of the solvent to make the 0.85% low reference solution.

B. Primary or secondary standards.

The primary or secondary standards in powder form must be weighed on an analytical balance to prepare the high concentrated reference solution when reference tablets are not available. It will be necessary to measure fractional volumes of solvent to insure that the correct volume to prepare the high concentration reference solution. Volumes required are obtained by using pipetts and a 1 mL graduated tuberculin syringe. The low concentration reference solution is prepared by diluting 1mL of the high concentration reference solution with 0.35 mL of solvent; antibiotics require 0.41 mL of solvent.

Developer

Prepare the developer fresh before developing the plate. Make only the volume needed for the days analyses. After the developer and plates have been added to the polyethylene bag allow the system to reach equilibrium for approximately 15 minutes. The separation will be improved and the spots less distorted.

Detector Solutions

The spots produced by the rapid TLC screening method may be detected by UV for all the TB drugs except ethambutol and streptomycin sulfate which do not have suitable groups for detection at 254 nm. Ethambutol requires staining with a solution of iodine to make the spots visible in ordinary light. Streptomycin sulfate is an amino glycoside and can be detected either by iodine staining or by reacting the developed spots with ninhydrin. The necessary staining solution is a mixture of iodine and potassium iodide. Most drugs are detectable by iodine-KI staining, so this method can be used when either UV or electricity is unavailable.

Iodine-potassium iodide solution

The iodine solution is prepared as follows:

The equipment needed is:

2 graduated cylinders with glass stoppers, 250 mL volume
1 actinic (brown) glass bottle with stopper, 500 mL

Wear plastic or rubber gloves and protective clothing (iodine can stain).

The following reagents are required for a single preparation of the iodine-KI solution:

8 g of potassium iodide
32 g of crystalline iodine
300 mL of 95% ethanol
25 mL of concentrated hydrochloric acid
81 mL of distilled water

Procedure: The iodine-KI solution is prepared by mixing 2 solutions.

Solution 1:

Dissolve 8 g (approximately one half teaspoon) of potassium iodide in a 250 mL graduated cylinder by adding 6 mL of water. Add 200 mL of 95% ethanol after the potassium iodide has dissolved, . Dissolve 32 g (approximately one and one half teaspoons) of crystalline iodine to this solution.

Solution 2:

Add 75 mL of distilled water to another 250 mL graduated cylinder,. Carefully add 25 mL of concentrated hydrochloric acid. Use caution when adding the acid slowly to the water. Use rubber gloves to prevent any burns. Add 100 mL of 95% ethanol to the acid solution. Mix this solution well.

Final solution:

Make the final iodine-KI solution by combining solutions 1 and 2 in a 500 mL brown glass bottle and cap the bottle tightly. This solution is stable and can be used over a period of several months if properly sealed. Replace the solution when excessive crystals of iodine form.

Ninhydrin solution.

Prepare the ninhydrin solution in a 25 mL glass stopper graduated cylinder . Add 25 mL of acetone to the cylinder , then add 0.1g of ninhydrin. Use care when preparing the solution by using rubber gloves because ninhydrin reacts immediately with the skin forming a black spot. Wash with a large amount of water if some of the solution has touched the skin. It is not necessary to prepare large quantities as the solution is stable and can be stored in dark bottles.

Transfer approximately 10 mL of the ninhydrin solution to the staining bag (same type as used for iodine staining). Dip the developed plate into the ninhydrin solution until the solution covers the spots. After drying and the acetone odor gone, the stained spots can be developed by heating for a short time (10 minutes) in an oven at 100E C.

Acknowledgment

Special thanks and appreciation is expressed to Jennifer Chan and W.Michael Johnson for their work in validating these methods and improving the procedures.

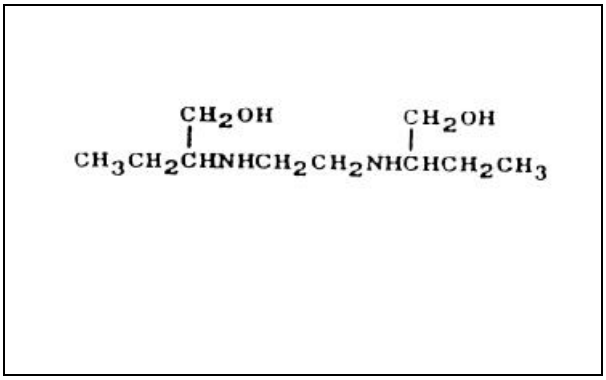
References

1. Tape/slide presentation "Training for Rapid Screening of Drugs by TLC" by A. S. Kenyon, P. E. Flinn, and T. P. Layloff has been developed at the Division of Drug Analysis, Food and Drug

Administration. Information on the availability of this presentation can be obtained through the Director, Division of Drug Analysis, FDA, St. Louis, MO.
2. "Rapid Screening of Pharmaceuticals by Thin-Layer Chromatography: Analysis of Essential Drugs by Visual Methods" by A. S. Kenyon, P. E. Flinn, and T. P. Layloff, Journal of AOAC International, 78(1),41-49,1995
3. "A Simplified TLC System for Qualitative and Semi-Quantitative Analysis of Pharmaceuticals" by P. E. Flinn, A. S. Kenyon, and T. P. Layloff, Journal of Liquid Chromatography, 15(10), 1639 (1992).

Ethambutol HCl
100 and 400 mg tablets

Structure



Molecular Formula & Mass: $C_{10}H_{26}Cl_2N_2O_2$ - 277
Category: Antibacterial (tuberculostatic)

Preparation of the sample solution:
Analytical balance available:

Prepare the sample solution by weighing an aliquot of the drug. Follow the procedure described in the previous section. Determine the weight of the drug and add solvent to produce a concentration of 2 mg/mL. Measure the volume accurately using a combination of pipetts plus a 1 mL graduated tuberculin syringe for the fractional volumes. Pipetts are available in 1mL increments up to 10 mL. For example: You weighed 23 mg of the drug, then you would add 11.5mL of solvent to prepare a solution with a concentration of 2mg/mL. (Use a 10 and 1mL pipetts and the 0.5 mL is measured by a 1mL graduated syringe).

NOTE: The above procedure applies to all of the TB drugs using the aliquot method.

Analytical balance not available.

The tablets listed below are representative of different drug content. Adjust the volumes accordingly for different composition. Always use volumes in full mL so that fractional volumes is not required. Most drugs have their contents in multiples of 5 which give a whole number for the concentration. Dissolve the sample in volumes which do give you a whole number for the concentrated solution. An aliquot of the concentrated solution is diluted to obtain the proper concentration for the TLC.

100 mg tablet:

Grind 1 tablet and dissolve in 10 mL of methanol which makes a solution having a

concentration of 10 mg/mL. Concentration of the required solution = 2 mg/mL to represent a 100% solution. Take 1 mL of the 10 mg/mL solution and add 4 mL of methanol to make the required concentration of 2mg/mL.

400 mg tablet:

Grind 1 tablet and dissolve in 25 mL of methanol. Concentration of the solution =16 mg/mL. Add 7 mL of methanol to 1 mL of the 16 mg/mL solution to make a final concentration equal to 2 mg/mL.

Preparation of standard or reference solutions:

Reference solutions are prepared depending on the availability of reference compounds. Reference tablets are available. The tablets contain a predetermined weight of the drug which when dissolved in 5 mL of the solvent will produce a solution concentration representing 115% of the sample solution. No weighing is required. Weighing is required when the reference compound is not available in tablet form. The reference solutions then must be prepared using either primary or secondary standards.

Preparation of the high standard when no reference tablets are available:

The high concentration limit is 115%; therefore the concentration of high standard = (2 mg/mL X 1.15) = 2.3 mg/mL. Weigh approximately 21 mg of standard. If you weighed 21.7 mg of standard, dissolve it in: (21.7 mg)/(2.3 mg/mL) = 9.4 mL of methanol.

Low standard:

The low limit is 85%; the concentration of low standard = (2 mg/mL) X 0.85 = 1.7 mg/mL. Dilute 1 mL of high standard to 1.35 mL by adding 0.35 mL of methanol= 1.35). This low reference solution is always prepared by the same procedure regardless of the reference source.

Spotting:

Spot on the TLC plate as follows:

Sample each of the solutions with a 3 : L capillary pipette and spot.

Left spot low standard (85%)

Center spot 100% sample

Right spot high standard (115%)

Development:

Mix 25 mL of methanol and 0.38 mL of concentrated ammonium hydroxide. Add 24 mL this mixture to the TLC development bag. Develop until the solvent front reaches within 1 cm of the top of the TLC plate.

Detection:

UV:

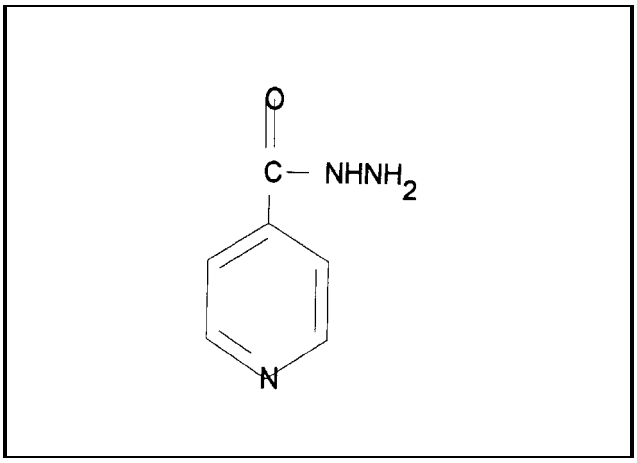
The spots are not visible under UV.

Iodine stain:

Dip the plate in the iodine-KI solution in the detection bag. Allow the plate to dry and observe the size and intensity of the spots.

Isoniazid
300 and 100 mg tablets

Structure:



Molecular Formula & Mass: $C_6H_7N_3O$ - 137.15
Category: Antibacterial (tuberculostatic)

Preparation of sample solution:
Analytical balance available.

Prepare the sample solution by weighing an aliquot of the drug. Follow the procedure described in the previous sections. Determine the weight of the drug and add solvent to produce a concentration of 0.5mg/mL. The volumes are measured accurately by using a combination of pipetts plus a 1 mL graduated tuberculin syringe for the fractional volumes. Pipetts are available in 1mL increments up to 10 mL. For example: You weighed 5.25 mg of the drug, then you would add 10.5mL of solvent to prepare a solution with a concentration of 0.5mg/mL. (Use a 10 mL pipette and measure the 0.5 mL by a 1mL graduated tuberculin syringe).

Analytical balance not available.

The entire dosage form is used with the declared drug content taken as the weight of the sample. The tablet contents have many different dosages, and the ones described are representative. All volumes must be accurately measured by pipettes.

<u>300 mg tablet</u>

Grind to a fine powder 1 tablet in a polyethylene bag and dissolve in 50 mL of methanol. The solution concentration 300 mg/50 mL = 6 mg/mL. The required concentration of the sample solution representing 100% is 0.5 mg/mL. Take 1 mL of the 6 mg/mL solution and add 11 mL of methanol to make 12 mL of solution which makes a final concentration of 0.5mg/mL.

<u>100 mg tablet</u>

Grind to a fine powder 1 tablet and dissolve in 25 mL of methanol. The concentration of

the solution is 100 mg/25 mL = 4 mg/mL. The required concentration of the sample solution representing 100% is 0.5 mg/mL. Take 1 mL of the 4 mg/mL solution and add 7 mL of methanol to make 8 mL of solution with a concentration of 0.5mg/mL.

Preparation of standards solutions:

Reference solutions are prepared depending on the availability of reference compounds. The reference materials may be either in the form of reference tablets or powders of primary/secondary standards. Reference tablets may be available containing a predetermined weight of the drug which when dissolved in 5 mL of the solvent produces a solution concentration representing 115% of the sample solution. No weighing is required.

Weighing is required when the reference compound is not available in tablet form. The reference solutions must be prepared using either primary or secondary standards.

<u>High standard solution:</u>

1. Reference tablet available.

The reference tablet contains 2.88 mg of isoniazid which when dissolved in 5 mL of methanol produces a solution having a concentration of 2.88mg/5 mL equal to 0.576 mg/mL which is 115% of the sample concentration. Drop one reference tablet into a vessel and add 5 mL of the solvent. No weighing is needed.

2. Reference material in the powder form (primary or secondary).

A reference solution having a concentration of 0.576 mg/mL is required. Weigh approximately 4-5 mg of powdered standard. For example: you weighed 4.2 mg of standard, dissolve it in: (4.2mg)/(0.575 mg/mL) = 7.3 mL of methanol. Measure the volumes by pipettes and a 1 mL graduated tuberculin syringe. This makes the high standard solution concentration equal to 0.575 mg/mL equal to 115% .

<u>Low standard:</u>

The low limit is 85%; therefore the concentration of the low standard = (0.5 mg/mL) X 0.85 = 0.425 mg/mL. Dilute 1 mL of high standard to 1.35 mL by adding 0.35 mL of methanol (0.575/0.425 = 1.35). The low standard is always prepared by taking 1 mL of the high and adding 0.35 mL of the solvent when the high solution represents 115% of the sample.

Spotting:

Spot on the TLC plate as follows:

Sample each of the solutions with a 3 : L capillary pipette and spot.

Left spot low standard (85%)
Center spot sample (100%)
Right spot high standard (115%)

Developer:

Mix 13 mL of methanol, 17 mL of acetone and 1 mL of concentrated ammonium hydroxide. Add 24 mL of this mixture to the TLC development bag. Develop until the solvent front reaches within 1 cm of the top of the plate.

Detection:

<u>UV:</u>

Dry the plate and observe under UV light (254 nm). Observe the size and intensity of the spots or stain with iodine when no UV available..

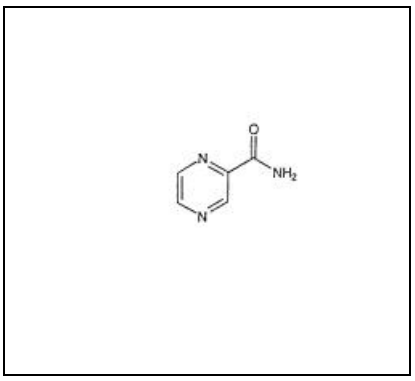
<u>Iodine stain:</u>

Dip the plate in the iodine-KI solution in the detection bag. Allow the plate to dry and observe the size and intensity of the spot.

Pyrazinamide

400 mg tablet

Structure:



Molecular formula & mass: $C_5H_5ON_3$--123.11
Category: Antibacterial (tuberculostatic)

Preparation of the sample solution:

Analytical balance available.

Prepare the sample solution by weighing an aliquot of the drug. Follow the procedure described in the previous sections. Determine the weight of the drug and add the volume of solvent to produce a concentration such of 1mg/mL. The volumes must be measured accurately using a combination of pipetts plus a 1 mL graduated tuberculin syringe for the fractional volumes. Pipetts are available in 1mL increments up to 10 mL. For example: You weighed 10.5 mg of the drug, then you would add 10.5mL of solvent to prepare a solution with a concentration of 1.0 mg/mL. (use a 10 mL and a 1mL pipetts and the 0.5 mL is measured by a 1mL graduated syringe).

Analytical balance not available.

Grind 1 tablet to a fine powder in a small polyethylene bag and insert the bag and contents into a suitable vessel and add 50 mL of methanol. Shake vigorously for at least 1 minute to dissolve the powder. The concentration of this solution is = 400/50= 8 mg/mL. The required concentration for the sample solution representing 100 % solution is 1 mg/mL. The concentrated solution must be further diluted. Take 1 mL of the 8 mg/mL solution and add 7 mL of methanol which will prepare the concentration of 1 mg/mL. The volume of solvent needed must be adjusted to produce the proper concentration.

Preparation of standard solutions:

Reference solutions are prepared depending on the availability of reference compounds. The reference materials may be either in the form of reference tablets or powders of primary/secondary standards. Reference tablets may be available containing a predetermined weight of the drug which when dissolved in 5 mL of the solvent produces a solution concentration representing 115% of the sample solution. No weighing is required.

Weighing is required when the reference compound is not available in tablet form. The reference solutions then must be prepared using either primary or secondary standards.

Reference solutions are prepared depending on the availability of reference compounds.

Reference tablets are available in many cases. These tablets contain a predetermined weight of the drug which when dissolved in 5 mL of the solvent will produce the high reference solution concentration representing 115% of the sample solution. No weighing is required.
Weighing is required when the reference compound is not available in tablet form. The reference solutions then must be prepared using either primary or secondary standards.

Preparation of the High Standard:

1. Reference tablet available.

The reference tablet for pyrazinamide contains 5.75 mg. Add one reference tablet to a vessel and add 5 mL of methanol to prepare a solution having a concentration of 5.75mg/5mL equal to 1.15 mg/mL. This represents 115% of the sample concentration.

2. Reference material available as a powder.

Weigh approximately 10 mg of the standard. For example you weighed 9.7 mg, then the volume of solvent added would be 9.7 mg/1.15 mg/mL = 8.43 mL of methanol. This will make a final concentration of 1.15 mg/mL which will represent the 115% solution.

Preparation of the low standard:

Dilute 1 mL of the high standard to 1.35 mL by adding 0.35 mL of methanol.(1.15/0.85) = 1.35 which is the high concentration divided by the low concentration).

Spotting:

Spot on the TLC plate as follows:

Sample each of the solutions with a 3∶L pipette and spot.

Left spot low standard (85%)
Center spot Sample (100%)
Right spot high standard (115%)

Development:

Mix 13 mL of methanol, 17 mL of acetone and 1 mL of concentrated ammonium hydroxide. Add 24 mL of this mixture to the TLC development bag. Develop until the solvent front reaches within 1 cm of the top of the plate.

Detection:

UV:

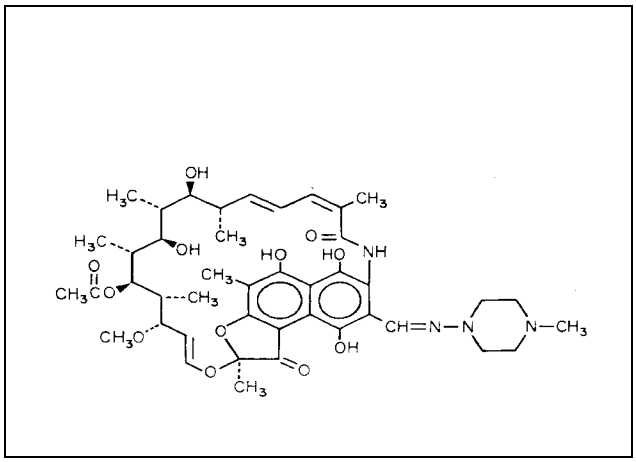
Dry the plate and observe under UV light (254 nm). Observe the size and intensity of the spots or stain with iodine when no UV available.

Iodine stain:

Dip the plate in the iodine-KI solution in the detection bag. Allow the plate to dry and

Rifampicin
150 mg capsules

Structure:



Molecular Formula & Mass: $C_{43}H_{58}N_4O_{12}$ - 822.96
Category: Antibacterial, (tuberculostatic)

Preparation of the sample solution:

The dosage forms of rifampin are supplied in many different quantities of the drug. The procedure described here is to serve as representative. Weights and volumes need to be adjusted for other dosages. Solutions are prepared by two different methods.

Analytical balance available.

Prepare the sample solution by weighing an aliquot of the drug. Follow the procedure described in the previous sections. Determine the weight of the drug in the aliquot and add solvent to produce a concentration such as 1mg/mL. The volumes must be measured accurately by using a combination of pipetts plus a 1 mL graduated tuberculin syringe for the fractional volumes. Pipetts are available in 1mL increments up to 10 mL. For example: You weighed 10.5 mg of the drug, then you would add 10.5mL of solvent to prepare a solution with a concentration of 1.0 mg/mL. (use a 10 mL pipette and the 0.5 mL is measured by a 1mL graduated tuberculin syringe).

Analytical balance not available.

Grind 1 tablet to a fine powder in a small polyethylene bag and insert the bag and contents into a suitable vessel and add 25 mL of methanol. Shake vigorously for at least 1 minute to dissolve the powder. The concentration of this solution is = 150/25= 6 mg/mL. The required concentration for the sample solution representing 100 % solution is 1 mg/mL. The concentrated solution must be further diluted. Take 1 mL of the 6 mg/mL solution and add 5 mL of methanol to prepare the proper concentration of 1 mg/mL.

Preparation of standard solutions:

Preparation of standard solutions:

Reference solutions are prepared depending on the availability of reference compounds. The reference materials may be either in the form of reference tablets or powders of primary/secondary standards. Reference tablets may be available containing a predetermined weight of the drug which when dissolved in 5 mL of the solvent produces a solution concentration representing 115% of the sample solution. No weighing is required. Weighing is required when the reference compound is not available in tablet form. The reference solutions then must be prepared using either primary or secondary standards.

Preparation of the High Standard:

1. Reference tablet available.

The reference tablet for rifampin contains 5.75 mg. Add one reference tablet to a vessel and add 5 mL of methanol to prepare a solution having a concentration of 5.75mg/5mL equal to 1.15 mg/mL. This represents 115% of the sample concentration.

2. Reference material available as a powder.

Weigh approximately 10 mg of the standard. For example you weighed 9.7 mg, then the volume of solvent added would be 9.7 mg/1.15 mg/mL = 8.43 mL of methanol. This will make a final concentration of 1.15 mg/mL which will represent the 115% solution.

Low standard:

The low limit is 85%; therefore the concentration of the low standard = (1.0 mg/mL) X 0.85 = 0.85 mg/mL. Dilute 1 mL of high standard to 1.35 mL by adding 0.35 mL of methanol (1.15/0.85 = 1.35).

Spotting:

Spot on the TLC plate as follows:

Sample each of the solutions with a 3 : L capillary pipette ans spot.

Left spot	low standard (85%)
Center spot	100% sample
Right spot	high standard (115%)

Development:

Mix 13 mL of methanol, 17 mL of acetone and 1 mL of concentrated ammonium hydroxide. Add 24 mL of this mixture to the TLC development bag. Develop until the solvent front reaches within 1 cm of the top of the plate.

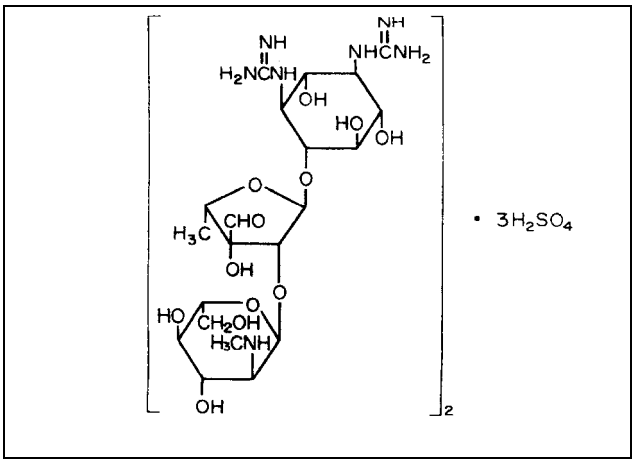
Detection:

Visible and UV:

Dry the plate and observe under white light. The maximum is at 445 nm. Observe the size and intensity of the spots. Rifampin has a strong absorption in the UV at 254 nm allowing excellent detection. The spots are better observed by UV when the stray lights of the room are eliminated.

Streptomycin sulfate
200 mg/mL injectable

Structure:



Molecular Formula & Mass: $(C_{21}H_{39}N_7O_{12})_2.3\ H_2SO_4$ - 1457.38
Category: Antibacterial (tuberculostatic)

Preparation of Sample Solutions:
NOTE: Streptomycin are handled differently from the other TB drugs. The solvents , developer, and detection are changed.
Analytical balance available.

Streptomycin is an aminoglycoside antibiotic. The drug in powder form is supplied in a vial labeled as streptomycin sulfate with a note saying that the vial contains 1 gram as the free base . The supplied drug is the sulfate listed as 1 gram of the free base which when reconstituted by adding 5 mL of water makes a solution having a concentration of 200 mg/mL. Actually the vial contains a weight as the sulfate equal to 1 X 1457.38/1163.14 (the 1457.38/1163.14 which is the ratio of molecular weights between the sulfate and the free base) equal to 1.2530 grams. The reference compound is supplied as the sulfate so the drug may be analyzed directly by weighing an aliquot of the drug and dissolving in a volume of water to prepare a solution having a concentration of 5 mg/mL which represents the 100% sample solution.

Analytical balance not available.

The drug is supplied as the sulfate but listed as 1 gram of the free base and is to be reconstituted by adding 5 mL of water to make a concentration of 200mg/mL. In this case the solutions are prepared on a volume basis.

The TLC requires a solution concentration of 5 mg/mL. Take 1 mL of this concentrated solution and add 39 mL of water to prepare the final concentration representing the 100%. Those volumes must be accurately measured.

Preparation of standard solutions:

Preparation of the high reference solution

Reference tablets available.

Reference tablets of streptomycin sulfate are supplied containing 38 mg which is equivalent to a content of 30 mg expressed as the free base (38 X 1163.14/1457.38). There are two ways to prepare the high reference solution .

1. Analyzed as the sulfate.

. The reference tablets contain 38 mg as the sulfate. Add one tablet to a vessel and add a volume of solvent to prepare the solution. The needed volume is determined by dividing 38 by 6 to obtain the volume of water needed which is 6.33 mL. Use a pipette and a 1 mL graduated tuberculin syringe to measure the volumes. This solution represents 120% of the sample concentration.

2. Analyzed as the free base.

The reference solutions must be prepared as the free base when the sample was prepared as the free base on a volume basis. The reference tablet contains 30 mg as the free base(streptomycin). Add 1 tablet to a vessel and add 5 mL of water to obtain a solution with a concentration of 6 mg/mL; this solution represents 120% of the concentration of the sample solution.

Reference tablets not available.

1. Analyzed as the sulfate.

The reference solutions must be prepared from a powdered reference material which are in the sulfate form. Prepare a solution having a concentration of 6 mg/mL. Weigh approximately 38 mg of powder sulfate standard. If you weighed 39.8 mg of sulfate standard, dissolve in a volume (39.8 mg)/6.0 mg/mL = 6.63 mL of distilled water. This prepares the high standard solution concentration equivalent to 6 mg/mL.

2. Analyzed as the free base.

When the sample solution has been prepared as the free base on a volume basis, then the reference solutions must be prepared as the free base. Weigh approximately 30 mg of the reference material as the sulfate and convert the weight to a free base by multiplying the weight by the ratio of the molecular weights. For example you weighed 30 mg as the sulfate, then the weight as the free base would be 30 X 1163.14/1457.38 equal to 23.94 mg. The volume of water needed is 23.94mg/6 mg/mL or 3.99 mL to prepare a reference solution representing 120% of the sample solution.

<u>Low standard:</u>

The low limit for antibiotics is 85%; the concentration of the low standard is (5 mg/mL) X 0.85 = 4.25 mg/mL. The concentration ratio between the high and low standard is 6/4.25 =1.41. Dilute 1 mL of high standard to 1.41 mL by adding 0.41 mL of water.

Spotting:

Spot on the plate as follows:

Sample each of the solutions with a 3 : L capillary pipette and spot.

Left spot low standard (85%)

Center spot 100% sample

Right spot high standard (120%)

Development:

Mix 4 mL of concentrated ammonium hydroxide and 4 mL of distilled water in a container fitted with a stopper. Carefully add 12 mL of glacial acetic acid. The solution will become very hot and generate ammonia gas. Quickly stopper the container, shake well to dissolve the ammonia gas, and cool to room temperature. Add 12 mL of ethyl acetate to the cooled mixture. Add 22 mL of this mixture to the TLC development bag. Develop the plate until the solvent front reaches 1 cm from the top of the plate which will require longer development time than the other TB drugs. Allow the plate to dry until the odor of acetic acid cannot be detected.

Detection:

UV:

The spots are not visible in the UV.

Iodine stain or ninhydrin:

Dip the plate in the iodine-KI solution in the detection bag. Allow the plate to dry and determine the size and intensity of the spots as soon as the spots become visible. The spots fade rapidly due to the sublimation of the iodine.

Ninhydrin stain:

The ninhydrin solution is needed to stain some drugs which are not visible by either UV or iodine staining. Because this solution will be used only for that one class of drugs (aminoglycosides), prepare the solution as described in the beginning section. Allow the plate to dry after the development until no odor can be detected. Dip the plate into the ninhydrin solution, then remove and allow to dry. The color of the spots is developed by heating at 100EC for ten minutes. This staining method requires the plates to dry for a long period. Ninhydrine also stains the background and unless all remaining ammonium or acetic acid is removed, the spots may be masked. Spots stained by ninhydrin will be more stable than those stained by iodine, but it will take longer to develop and an oven will be required to develop the spots.

Fixed Drug Compositions(FDC):

Dosage forms of TB drugs are now being formulated to contain combinations of two or more components with different ratios know as Fixed Dosage Combinations(FDC). The FDC drugs are supplied in many different ratios of the separate drugs. The drugs must be separated by a sufficient distance to quantify any one component. Analysis of the individual component may require different sample solution concentrations because the components probably will not be the proper ratios for TLC. Drugs containing rifampin, isoniazid, and pyrazinamide are separated using a single developer consisting of methanol, acetone, and ammonium hydroxide (13/17/1) on a volume basis. This developer may be used for the single or for the multiple component drugs. If ethambutol is present as the fourth compound, this same developer can be used since ethambutol will not be UV sensitive. Ethambutol must be analyzed by a different developer.

Isoniazid + Ethambutol

400 mg + 150 mg tablet

Structures:

See the structures of the individual drugs.

Molecular Formula and Masses:
Isoniazid--$C_6H_7N_3O$--137.1
Ethambutol--$C_{10}H_{26}Cl_2N_2O_2$---277
Category: Antibacterial (tuberculostatic)

The above combination is a representative of several different ratios.

Preparation of sample solutions:

The preparation of sample solutions depend on the availability of an analytical balance.

1. Analytical balance available.

Weigh the tablet/capsule and determine the fraction of each drug present. Grind the tablet to a fine powder. Weigh an aliquot of this powder and add a volume of methanol necessary to produce the required TLC concentration for each drug; the concentration required for isoniazid is 0.5mg/mL and the concentration for ethambutol is 2.0mg/mL. It is necessary to make two separate weighings of aliquots because of the broad differences in content. These solutions represent 100%.

2. Analytical balance not available.

The required concentrations for suitable TLC detection are 0.5 mg/mL for isoniazid and 2.0 mg/mL for ethambutol. Grind 1 tablet to a fine powder in a polyethylene bag and transfer bag and contents to a vessel. Add a volume of methanol to dissolve the drug which will produce a solution having different concentrations of each drug. Calculate the concentration for each drug by dividing the declared drug content by the volume added. Use volumes that are multiples of 5 to produce a solution having a whole number as the concentration. Take 1 mL of the concentrated solution and dilute to prepare the final solution. Each drug require different volumes of solvent to prepare the proper concentrations.

Preparation Reference Solutions:

The reference solutions are prepared either by using the reference material as a fixed content reference tablet or by weighing a powder of the reference compound either as a primary or secondary standard.

High standards:

The high standard solutions are prepared as described for the individual drug. A high standard for each drug is required; the concentrations needs to be 115% greater than the sample solution concentration. Follow the procedures described for the preparation of the individual drug high reference solution when either an analytical balance is or is not available.

Low standards:

The low standard limit is 85% of the sample concentration. The concentrations of both standards are achieved by taking 1 mL of each high standard and adding 0.35 mL of methanol.

Spotting:

Spot the solutions (sample and references) of one drug on one plate and the solutions of the other on the second plate.

Spot as follows:

Sample each of the solutions with a 3 : L capillary pipette and spot.

Left- low standard of one drug (85 %)

Center- sample which will contain both drugs (100%)

Right- high standard of one drug (115%)

Development:

The developer for isoniazid is a mixture of methanol, acetone, and ammonium hydroxide (13/17/1). Add 24 mL of this mixture to the polyethylene bag. The developer for the ethambutol is a mixture of 25 mL of methanol and 0.38 mL of concentrated ammonium hydroxide. Transfer 24 mL of this solution to the polyethylene bag. Develop the spots for each in separate bags with the different developers until the solvent fronts in each case reach 1 cm below the top of each plate.

Detection:

The isoniazid can be detected by UV at 254 nm but the ethambutol can not be detected due to the lack of absorbing groups. Both can be detected by dipping in an iodine solution and allowing the excess iodine to sublime. Dip the plate into the iodine-KI solution and allow the plate to dry.

Ethambutol is not visible in the UV and must be stained with iodine solution.

Rifamate
(Rifampin and Isoniazid)
300 mg rifampin and 150 mg isoniazid capsule
150 mg rifampin and 75 mg isoniazid
150 mg rifampin and 150 mg isoniazid

Structure:

See structures for rifampin and isoniazid.

Molecular Formulae & Masses:
Rifampin : $C_{43}H_{58}N_4O_{12}$ - 823.0
Isoniazid: $C_6H_7N_3O$ - 137.1
Category: Antibacterial (tuberculostatic)

Preparation of sample solutions:

The rifampin and isoniazid tablets/capsules are supplied in many different content ratios of the drugs. The compositions shown above are only representative. The quantity needed depends on the amounts of each drug in the dosage form. The preparation method for the sample solution depends on the availability of an analytical balance.

1. Analytical balance available.

Weigh the tablet or the capsule with and without contents. Determine the fractional content of each component based upon the declared content of each drug. Grind the tablet to a fine powder and empty into a vessel. It is not necessary to grind the contents of capsules. Weigh an aliquot and add a volume of solvent needed to prepare a sample solution representing 100%. The concentration needed for rifampin is 1.0 mg/mL and isoniazid needs a concentration of 0.5 mg/mL. It is possible to make only one solution if the ratio between the contents of the two drugs is 2 to 1. Otherwise two separate solutions are necessary, one for the rifampin and one for the isoniazid. For example: The tablet containing 300 mg of rifampin and 150 mg of isoniazid would require only one solution. Other content ratios require two sample weighings and separate solutions Weigh out and aliquot of the ground powder and multiply the weight by the fractional content for each drug to obtain the actual weight. Add a volume of methanol to prepare the sample solution with a concentration needed for the TLC. Measure the volumes with pipettes and a 1 mL graduated tuberculin syringe.

2. Analytical balance not available.

Place 1 tablet into a small polyethylene bag and grind to a fine powder. Add the bag and contents to a vessel and add a volume of methanol needed to dissolve the entire contents. The volume of methanol needed should be a multiple of 5 to prepare a concentrated solution having a whole number for its concentration such as 6mg/mL. If your tablet contained 300 mg of rifampin,

add 50 mL of methanol. This solution is too concentrated for the TLC. Take 1 mL of the concentrated solution and sufficient methanol to prepare a sample solution of the proper concentration. This solution represents the 100% solution. Only one solution is necessary for this composition. Two solutions are required if the ratios are other than the 2 to 1 ratio. In this case, take the concentrated solution and dilute for each drug to prepare the two solutions.

The different contents of the active drugs require different procedures for preparation of the sample solutions.

Preparation of the standard solutions:

1. Reference tablets available:

Reference tablets for rifampin and isoniazid are available in either single component or combination of both drugs. The single component tablets contain 5.75 mg of rifampin and 2.88 mg of isoniazid. The multiple component tablets contain the same quantities of each drug as listed above. It is preferred to use the multiple component tablets when available because only 5 mL of solvent is used to prepare the 115%reference solution so that less excipients would be present. You would have double the quantity of excipients if you used two individual tablets. One reference tablet is added to a vessel and 5 mL of methanol added. It is not necessary to grind reference tablets because they have been formulated to disperse when the solvent is added.

2. Reference tablet not available

The high reference solution is prepared by weighing a powder of either a primary or secondary standard. The high limit of both reference materials is 115%; therefore the concentration of rifampin is 1 mg/mL X 1.15 =1.15 mg/mL. Weigh approximately 7-8 mg of the standard rifampin. For example you weighed 7.5 mg then dissolved that quantity in: (7.5 mg/ 1.15 mg/mL = 6.52 mL of methanol. Then the high standard has a concentration of 1.15 mg/mL.

The concentrated solution needed for isoniazid is 0.5 mg/mL X 1.15 = 0.575 mg/mL. Weigh approximately 4-5 mg of the isoniazid standard. If you weighed 4.2 mg of the isoniazid, dissolve it in 4.2 mg/0.575 mg/mL = 7.3 mL of methanol. The concentration of the high standard is 0.575 mg/mL

Low standard:

The concentration of the low standard is 85%. Take 1 mL of each of the high standards and add 0.35 mL of methanol to each of the high standards.

Spotting the solutions:

Sample each of the solutions with a 3:L capillary pipette and spot. Spot the drugs on separate plates.

Plate #1. Spot the 85% rifampin solution on the left position.

Spot the sample representing 100% in the center position. Spot the 115% solution of rifampin on the right position.

Plate #2. Spot the 85% isoniazid solution on the left position. Spot the sample solution (same solution as above containing both drugs) representing 100% in the center. Spot the 115% solution of isoniazid on the right position.

Both plates may be developed simultaneously or separately.

Developing solvent:

Mix 13 mL of methanol, 17 mL of acetone, and 1 mL of ammonium hydroxide. Add 24 mL of this mixture to the polyethylene bag. Develop until the solvent front reaches to within 1 cm of

top of the plate.

Detection:

Dry the plates. The intensity of the spots of rifampin may be detected under visible light however, better and easier detection is possible under UV since rifampin has a very strong absorption at 254 nm. The elimination of room lighting enhances the intensities. Look for differences in intensities.

The spots of isoniazid are observed under UV at 254 nm. Observe the difference in intensities.

Rifampin + Isoniazid + Pyrazinamide

150 mg + 75 mg + 400 mg tablet
150 mg + 150 mg + 500 mg tablet

Structure: See structures for the individual drugs.
Molecular Formula and Masses:
Rifampin : $C_{43}H_{58}N_4O_{12}$--823.0
Isoniazid: $C_6H_7N_3O$-- 137.1
Pyrazinamide: $C_5H_5ON_3$--123.11
Category: Antibacterial (tuberculostatic)

Preparation of sample solutions:

1. Analytical balance available:

Weigh the tablet or the filled capsule and weigh the empty capsule. Determine the fractional content of each component based upon the declared amount of each drug. Grind the tablet to a fine powder and empty into a vessel. It is not necessary to grind the contents of capsules. Weigh an aliquot of this finely ground powder, and determine the weight of the drug by multiplying the sample weight by the fractional content; add a volume of solvent needed to prepare a sample solution representing 100%. The concentration needed for rifampin is 1.0 mg/mL, isoniazid is 0.5 mg/mL, and pyrazinamide is 1.0 mg/mL. The ratios of these three components probably will not be the proper ratios for one solution which makes it necessary to prepare more than one solution to have proper concentrations for the TLC. As many as three separate solutions may be required.

2. Analytical balance not available:

Grind 1 tablet to a fine powder in a small polyethylene bag and transfer the contents to a vessel and add 50 mL of methanol. If the tablets contains 150 mg rifampin, 75 mg isoniazid, and 400 mg pyrazinamide, the solution would have a concentration of 3mg/mL for rifampin, 1.5 mg/mL for isoniazid, and 8 mg for pyrazinamide. Take 1 mL of the concentrated solution mixture and add 2 mL of methanol to make a solution having a concentration of 1mg/mL of rifampin and 0.5 mg/mL of isoniazid. Take another 1 mL of the concentrated mixture and add 7 mL of methanol to make a solution concentration of 1 mg/mL of pyrazinamide. Only two solutions of the concentrated solution were needed with these rations. Three solutions would be required if the isoniazid was in a different ratio. One mL of the concentrated solution is always taken and diluted accordingly.

Since these drugs are available in a wide range of content combinations, it is necessary to prepare the solution for a single drug. Each drug will have a different concentration when the initial solution is prepared. Take 1 mL of the concentrated solution for each drug and add the volume of solvent to prepare the individual solutions. Use the declared content of each drug as the sample weight, and determine the volumes needed to prepare the correct concentration.

Preparation of standard solutions:
High reference solution:

1. Reference tablets available:

Reference tablets for rifampin and isoniazid are available in either the single component or

the combination of both drugs, and pyrazinamide is available as a single component. The single component tablets contain 5.75 mg of rifampin and 2.88 mg of isoniazid. The multiple component tablets contain the same quantities of each drug as listed above. Reference tablets of pyrazinamide are available containing 5.75 mg. Use the multiple component reference tablets when available because only 5 mL of solvent is used to prepare the 115%reference solution so that less excipients would be present. The quantity of excipients would be doubled if two individual reference tablets were used. One reference tablet is added to a vessel and 5 mL of methanol added. It is not necessary to grind reference tablets because they have been formulated to disperse when the solvent is added.

2. Reference tablet not available

The high reference solution is prepared by weighing the powder form of a primary/ secondary standard. The high limit for the reference solutions are 115% of the sample concentration; therefore the concentration of rifampin is 1 mg/mL X 1.15 =1.15 mg/mL. Weigh approximately 7-8 mg of the standard rifampin. For example you weighed 7.5 mg then dissolved that quantity in: (7.5 mg/ 1.15 mg/mL = 6.52 mL of methanol. Then the high standard has a concentration of 1.15 mg/mL.

The solution concentration needed for isoniazid is 0.5 mg/mL X 1.15 = 0.575 mg/mL. Weigh approximately 4-5 mg of the isoniazid standard. If you weighed 4.2 mg of the isoniazid, dissolve it in 4.2 mg/0.575 mg/mL = 7.3 mL of methanol. The concentration of the high standard is 0.575 mg/mL and represents 115% of the sample concentration.

The required solution concentration for pyrazinamide is 1.0 mg/mL. Weigh the standard and add the volume of methanol necessary to prepare a solution with a concentration of 1.15mg/mL.

Low standard solution:

The concentration of the low standard is 85% of the concentration of the sample solution. Take 1 mL of each of the high standard solutions and add 0.35 mL of methanol to each of the high standard solutions.

Spotting the solutions:

Sample each of the solutions with a 3 : L capillary pipette and spot.

There are two possible spotting procedures, namely: 1. No reference tablets available and 2. Reference tablets available.

Condition 1. Plate 1

Left spot- low reference of rifampin (85%)
Center spot- sample solution(100%)
Right spot- high reference of rifampin (115%)
Plate 2
Left spot- low reference of isoniazid(85%)
Center spot- sample solution(100%)
Right spot- high reference of isoniazid(115%)

Develop these 2 plates in the developing solvent.

Prepare a third plate.

Left spot-low reference of pyrazinamide(85%)
Center spot- sample solution (100%)

Right spot- high reference of pyrazinamide (115%).

Develop as before.

Condition 2. Plate 1

Left spot- low reference of rifampin and isoniazid(85%)

Center spot- sample solution(100%)

Right spot- high reference of rifampin and isoniazid(115%)

Plate 2

Left spot- low reference of pyrazinamide(85%)

Center spot--- sample solution(100%)

Right spot- high reference of pyrazinamide(115%)

Developing solvent:

Mix 13 mL methanol, 17 mL acetone, and 1 mL ammonium hydroxide. Add 24 mL of this mixture to the polyethylene developing bag. Develop until the solvent reaches 1 cm from the top of the plate.

Detection:

Dry the plates. The spots of rifampin can be visually detected by observing the spots in white light. The spots are better resolved under UV when the room or other stray lights are eliminated.

Spots of Isoniazid and pyrazinamide are detected by UV at 254 nm