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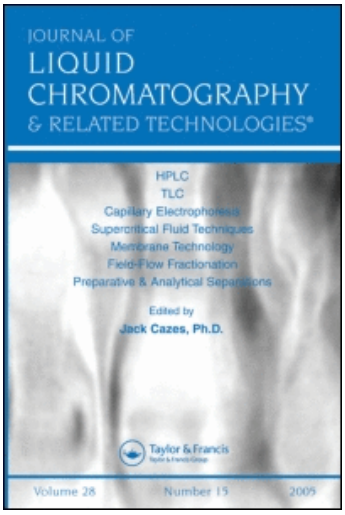

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RAPID SCREENING OF TUBERCULOSIS PHARMACEUTICALS BY THIN LAYER CHROMATOGRAPHY

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ABSTRACT

A quick, economical, and reliable thin layer chromatography (TLC) method for rapid screening of tuberculosis pharmaceuticals is described. The methods are based on the use of a portable kit involving grinding of samples, development with mobile phase, and application of detection reagents in polyethylene bags, or detection under ultraviolet light. Sample zones are compared to high and low reference standards developed on the same layer to determine if the drug content is within the specification range. Detailed instructions are given for specific drugs for which the TLC analysis has been successfully applied.

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INTRODUCTION

A simplified, inexpensive technique, using silica gel plates or sheets developed in standard TLC chambers, and detection by dipping the developed layer in iodine solution, was described earlier[1] for detecting and estimating 10 commonly used drugs in order to determine if the content of pharmaceuticals containing the drugs fall within specification limits. A portable system for performing rapid TLC screening of pharmaceuticals, using a polyethylene bag as the developing chamber with the iodine detection method, was then designed for use in field-type operations; its use reported analysis of essential drugs[2] and detection of diethylene glycol/ethylene glycol contamination of glycerin and glycerin-based raw materials.[3] This paper describes specific procedures for the application of this system for the rapid screening of tuberculosis (TB) pharmaceuticals that have been used successfully in underdeveloped countries by personnel having little training at non-laboratory, remote locations, such as ports of entry, pharmacies, distribution centers, or areas lacking resources for more complicated methods of analysis. The technique reduces the need for other analytical methods that are more costly and time consuming, and require highly trained operators.

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EXPERIMENTAL

The procedures described below are for screening a single content of each particular drug. Two reference solutions representing the upper and lower dosage limits (85% and 115 or 120% of the correct value), are spotted on the plate in 3.0 μL aliquots with a sample solution representing 100% spotted between the standards. After development, the zones are examined visually under ultraviolet (UV) light, or in visible light after detection by iodine. The drug is considered to be within specifications if the intensity of the sample zone lies between those of the reference zones. If the sample zone appears to be at or near the lower limit or outside of the range of the lower and upper zones, confirmation is obtained by TLC analysis of a pool of 10 samples, followed by further testing using an official method, if necessary. UV analysis, based on the USP 24 method for dissolution testing, is used as a confirmation method when applicable and when no interference was shown.

No further analysis is needed for those drugs that show concentrations within the specification range. Analysis of drugs, other than those specified, requires method development to determine a suitable mobile phase, and standard, and sample solution concentrations. For analyzing drugs with different contents than those listed, more or less solvent should be used to prepare a sample solution with the same concentration as described below, and the reference solutions should not be changed. Drugs formulated in liquid form rather than a solid, are

handled on a volume basis (mg/mL) and diluted as needed. The equipment costs are under $100 for the TLC method, and a basic technician can perform a test in less than one hour with a cost below $1 per test.

Experimental details for standard and sample preparation, and TLC analysis for specific antibacterial (tuberculostatic) drugs to which the TLC method applies, are contained in the Appendix.

Preparation of Sample Solutions

No analytical balance is required for sample preparation. A tablet is ground to a fine powder in a small polyethylene bag. The bag should be of sufficient strength (thickness) to prevent it from rupturing when crushing the tablet with a pestle. The contents of capsules are simply emptied into a vessel. The bag and powder are transferred to a suitable vessel (e.g., a beaker, flask, or bottle), and the proper volume of solvent is added and the vessel shaken vigorously to dissolve the powder in order to prepare a concentrated solution, from which the TLC sample solution is prepared by dilution. For example, if a 1.00 mg/mL solution is required and the stated value of the tablet is 200 mg of drug, the entire tablet is dissolved in 20.0 mL of solvent to make a 10.0 mg/mL solution, which is then diluted 1:10 (e.g., 1.00 mL solution + 9.00 mL of solvent) to prepare the TLC sample solution.

The solvent volume added to prepare the concentrated solution, must be known to be sufficient to completely dissolve the active drug; other ingredients in the tablet may not dissolve. The sample should be dissolved in a volume that gives a whole number for the concentrated solution. Volumes used should be in full mL values; most drugs have their contents in multiples of 5, which give a whole number for the concentration.

Preparation of Reference Standard Solutions

A reference standard tablet containing a predetermined quantity of the drug, is available for Rifampin by contacting Kayla Laserson.[4] A standard solution is prepared by dissolving a tablet in a fixed volume of solvent to prepare the high reference solution. Alternatively, a reference standard is weighed to at least 0.1 mg and diluted properly to prepare a primary standard solution. Secondary standards are less expensive and can be obtained from a previously analyzed sample, or from reputable chemical suppliers.

Reference tablets require no weighing for preparation of reference solutions. It is not necessary to grind reference tablets because they have been formulated to disperse when solvent is added. For all drugs except antibiotics, 5.00

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mL of solvent is added to the tablet in a suitable vessel, to obtain a solution equivalent to 115% when the sample is prepared to be 100%. For antibiotics, the tablet will produce a high solution with a concentration equivalent to 120%. The low reference solution (85.0% relative to the sample) is prepared by mixing 1.00 mL of the high reference solution, and adding 0.35 mL of solvent (0.41 mL for antibiotics).

Primary, or secondary standards in powdered form, must be weighed on an analytical balance, and fractional mL values measured with volumetric pipets and a 1 mL graduated tuberculin syringe, to prepare the high concentration reference solution. The low concentration reference solution is prepared by diluting 1.00 mL of the high concentration solution with 0.35 mL of solvent (0.41 mL for antibiotics).

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Thin Layer Chromatography

TLC is carried out using the portable kit supplied with plastic bags, holders, and all accessories required to perform the analysis ("Speedy TLC Kit," available from Granite Engineering Inc., #3 Thomas Court, Granite City, IL 62040). Volumes specified in this paper are suitable for a flat plastic development bag 10 cm wide, which require 15 mL of the mobile phase. Flat 10 cm plastic tubing can be obtained in rolls (006 gage), and bags are fabricated from the 10 cm tubing by using a bag sealer. Developing bags can be reused unless they begin to leak.

Merck plastic-backed silica gel 60 F254 sheets, or their equivalent, have been found to be satisfactory for the analyses described. Sheets 5 x 10 cm are required for use in the kit apparatus and can be cut from larger sheets. The presence of fluorescent indicator in the layer is necessary for detection of drugs under 254 nm UV light as black spots on a fluorescent green background. The specified mobile phase should provide the required separation for each analysis; if the mobile phase is changed, the R_f values of the zones, the separation obtained, and the development time will vary. Final spot positions should be between R_f of 0.2-0.8 for best results.

Samples and standards aliquots of 3.0 μL are applied using capillary pipets. The left zone is the low standard (85%), the center zone is the 100% sample, and the right zone is the high standard (115% or 120%). Before spotting, a pencil line is marked about 1 cm from the top of the layer to indicate the farthest advance of the mobile phase, and small points are marked on either side of the spotting locations about 2 cm from the bottom of layer. The initial zones are dried before development; if aqueous or partially aqueous solvents are used, several minutes will be needed for drying. Spots should be kept as small as possible.

The TLC frame, plastic bag, filter paper saturator strips, aluminum developing tray, and clamp and fishhook are assembled, and the mobile phase is added. The sheet is attached to the aluminum frame with the clip and lowered into the plastic bag with the fishhook. Paper clips are placed behind the sheet (between the sheet and the aluminum frame). The sheet is essentially suspended in space and is held only with the clip. The developing solvent will advance in a straight line. The sheet is allowed to stay in the bag without contacting the mobile phase for about 5 min to reach equilibrium, after which the plastic bag is pulled down to allow the mobile phase to contact the lower 1 cm of the layer.

Sheets are developed to within 1 cm of the top of the sheet with methanol-conc. ammonium hydroxide (25:0.38) (mobile phase a), methanol-acetone-conc. ammonium hydroxide (13:17:1) (b), or ethyl acetate-glacial acetic acid-conc. ammonium hydroxide-water (12:12:4:4) (c); 15 mL (18 mL for mobile phase c) is placed in the development bag. Mobile phase (c) is prepared by mixing 4.0 mL of ammonium hydroxide and 4.0 mL of distilled water in a container fitted with a stopper, adding 12 mL of acetic acid (care is required-the solution becomes very hot), quickly stoppering the container and shaking well to dissolve the generated ammonia gas, cooling to room temperature, and adding 12 mL of ethyl acetate. Pipets or graduated cylinders should be used for measuring the solvent volumes for mobile phase preparation. Fresh mobile phase should be prepared for each sheet because composition can change upon standing due to uneven evaporation of the solvent components. It may be acceptable to prepare enough mobile phase for use in a single day if storage is in a tightly sealed container. The development bag and back-to-back aluminum trays will accommodate two sheets at a time. The 5 min equilibration period before starting development improves the separation and produces spots that are less distorted. A photograph of the development apparatus and description of its use were presented by Kenyon et al.[3] Table 1 shows a listing of R_f values of the drugs in the various mobile phases. Rifampin quinone is a breakdown product of Rifampin that may be detected on chromatograms of products containing that compound.

The iodine staining solution is placed in a plastic bag, the sheets are dipped into the reagent and immersed, and they are then removed, and the zones observed after excess reagent is evaporated. The bag for detection is made by cutting the development bag ca. 12 cm above the seal and a slit in one side ca. 9 cm above the seal. A protective covering, such as cardboard or plastic film, is placed on a vertical surface to protect the surface from staining. The bottom of the bag is taped, with the top of the bag above the slit, to the vertical surface on top of the protective covering. A small, flat, rigid object is taped to the bag at the seal-point of the bag to act as a hinge to displace the iodine solution upwards. If enough stain solution is used, the TLC sheet can simply be dipped and removed for drying.

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Table 1. R_f Values of Drugs in the Three Mobile Phases

Drug	Mobile Phase (a)	(b)	(c)	Detection
Ethambutol	0.30	-	-	iodine
Isoniazid	0.62	0.40	-	UV
Pyrazinamide	0.58	0.50	-	UV
Rifampin	0.80	0.75	-	vis or UV
Rifampin quinone	0.65	0.65	-	vis or UV
Streptomycin	-	-	0.25	iodine

The three mobile phases are defined in the text.
vis = visible light.
− = data not available.

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The zones for all drugs can be detected, after drying the layer, by viewing fluorescence quenching under 245 nm UV in a TLC viewing box or unlighted room light, except for Ethambutol, Streptomycin, and Kanamycin, which require iodine staining. Most drugs are detectable using the iodine reagent, so this method is applicable when either UV light or electricity is unavailable. Battery operated UV lamps can be purchased. After detection, the sizes and intensities of the standard and sample zones are compared. The sample zone intensity should be between the intensities of the standards. Other criteria for an acceptable drug analysis include no additional, unexplained zones in the sample, and exact line up of standard and sample zones (identical R_f values).

The iodine-potassium iodide detection reagent is prepared as follows:

Solution 1. 8 g (ca. one-half teaspoon) of KI is dissolved in a 250 mL graduated cylinder by adding 6.0 mL of distilled water and then 200 mL of 95% ethanol, and 32 g (ca. 1.5 teaspoons) of crystalline iodine are added (plastic or rubber gloves should be worn because iodine can stain the skin). Solution 2. 75 mL of distilled water is added to another 250 mL graduated cylinder, 25 mL of conc. hydrochloric acid is added carefully and slowly (rubber gloves should be worn to prevent burns), 100 mL of 95% ethanol is then added, and the solution is mixed well. The detection solution is prepared by combining Solutions 1 and 2 in a brown (actinic) glass bottle. The solution should be tightly capped and is stable for several months; the solution is replaced when excessive crystals of iodine form. Detected zones fade rapidly due to sublimation of iodine.

About 20-50 mL of solution is placed in a staining bag, the sheet is dipped into the solution until the entire sheet is covered, and then the sheet is removed and dried. The solution is stable if stored in a dark bottle. The mobile phase must be completely removed prior to application of the reagent, or the background will be colored and mask the drug zones.

RESULTS AND DISCUSSION

The methods described for semiquantitative screening of TB drugs have not been subjected to a collaborative (round robin) test. None of the methods is official. The drugs selected are those that are currently being used worldwide for treatment of TB. Questions about the methods and the availability of a tape/slide training presentation should be addressed to Kayla Laserson.[4]

Dissolution testing has been performed for confirmation on a limited basis. These tests have demonstrated dissolution problems in several TB drug samples, which may indicate lack of bioavailability of these drugs for the TB patient. Dissolution testing, coupled with TLC, could give a comprehensive picture of the quality of TB drugs in countries surveyed.

TB drugs come in many different contents, and the methods described here are for only one specific content. Solution concentrations will have to be adjusted for analysis of other formulations. The TLC method gives a good estimate of whether the drug is the same one as listed on the label and if its content is the correct amount as specified. The method is not intended to replace any official compendium method. In addition to single-drug medications, fixed dose combinations (FDC) containing combinations of drugs, can also be analyzed by the method (see the Appendix below).

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Various safety considerations are addressed in the procedures described above. All chemicals should be considered to be toxic, and vapors or dust from them, should not be inhaled and contact with skin avoided by wearing protective clothing and gloves. All analyses should be performed in adequately ventilated areas, and flammable solvents should be kept away from flames or ignition sources. Local rules for disposal of chemicals should be followed.

Previous papers[5,6] described the use of these TLC-kit methods to screen Isoniazid and Rifampin single- and fixed-dose combinations from selected TB programs and pharmacies in Colombia, Estonia, India, Latvia, Russia, and Vietnam. All abnormal samples, and a 40% random sample of normals were further analyzed using confirmatory techniques. A substantial number of TB drugs from many countries, particularly FDC formulations, were found to be substandard (outside of 85-115% of the stated content). The TLC method was shown to be an effective, convenient, and inexpensive method for detection of these substandard drugs.

A training course was conducted in July, 2000 by the World Health Organization and the Centers for Disease Control in order to train participants from six African countries in the use of the TLC method, to provide the epidemiological context of TB in Africa for the participants to learn the importance of TB drug quality screening for effective TB control and prevention, and to screen TB drug samples from each participant's home country, as well as TB drug samples from clinics and the government hospital in Potchefstroom, South Africa. Thirty-eight TLC tests were conducted, of which only one sample, an FDC containing

Isoniazid and Thiaocetazone, was found to be substandard. Three TB drugs were found to be of low quality when utilizing UV confirmatory methods, and one TB drug was found to be of low quality when dissolution testing was performed. Unpublished results of these tests can be obtained from Kayla Laserson.[4]

APPENDIX

Single-Drug Dosage Forms

Ethambutol hydrochloride (100 and 400 mg tablets)

Sample solution. Analytical balance available: prepared by the aliquot method at a concentration of 2.00 mg/mL in methanol. Analytical balance not available: 100 mg tablet is dissolved in 10.0 mL of methanol (10.0 mg/mL) and this solution is diluted 1:5 (1.00 mL of solution + 4.00 mL of methanol) to prepare a 2.00 mg/mL TLC solution; 400 mg tablet is dissolved in 25.0 mL of methanol (16.0 mg/mL) and diluted 1:8 (1.00 mL of solution + 7.00 mL of methanol) to a concentration of 2.00 mg/mL.

Reference solutions. The high standard is prepared in methanol at 2.30 mg/mL (115%) and the low standard at 1.70 mg/mL (85%) by mixing 1.00 mL of high standard + 0.35 mL of methanol.

TLC analysis. The layer is developed with mobile phase (a) and zones detected using iodine-KI solution. Zones are not visible under UV light.

Isoniazid (100 and 300 mg tablets)

Sample solution. A 100 mg tablet is dissolved in 25.0 mL of methanol (4.00 mg/mL) and this solution is diluted 1:8 to prepare a 0.500 mg/mL TLC solution; 300 mg tablet is dissolved in 50.0 mL of methanol (6.00 mg/mL) and diluted 1:12 to a concentration of 0.500 mg/mL.

Reference solutions. The high standard is prepared in methanol at 0.576 mg/mL (115%) and the low standard at 0.425 mg/mL (85%).

TLC analysis. The layer is developed with mobile phase (b) and zones detected under UV light or by using iodine-KI solution.

Pyrazinamide (400 mg tablets)

Sample solution. A 400 mg tablet is dissolved in 50.0 mL of methanol (8.00 mg/mL) and this solution is diluted 1:8 to prepare a 1.00 mg/mL TLC solution.

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Reference solutions. No reference tablet available: the high standard is prepared in methanol at 1.15 mg/mL (115%) and the low standard at 0.850 mg/mL (85%).

TLC analysis. The layer is developed with mobile phase (b) and zones detected under UV light or by using iodine-KI solution.

Rifampin (150 mg capsules)

Sample solution. A 150 mg tablet is dissolved in 25.0 mL of methanol (6.00 mg/mL) and this solution is diluted 1:6 to prepare a 1.00 mg/mL TLC solution. For other dosage forms containing different quantities of the drug, weights and volumes are adjusted to prepare the 1.0 mg/mL solution.

Reference solutions. No reference tablet available: the high standard is prepared in methanol at 1.15 mg/mL (115%) and the low standard at 0.850 mg/mL (85%). Reference tablet available: the reference tablet contains 5.75 mg of Rifampin, which when dissolved in 5.00 mL of methanol produces the 115% solution at a concentration of 1.15 mg/mL.

TLC analysis. The layer is developed with mobile phase (b) and zones detected under UV light or in daylight (maximum absorbance 445 nm).

Streptomycin sulfate (200 mg/mL injectable)

Sample solution. The reference compound is supplied as the sulfate, so the drug is analyzed directly by weighing an aliquot of the drug and dissolving in a volume of water to prepare a solution having a concentration of 5.00 mg/mL, which represents the 100% solution. Analytical balance not available: The 100% solution is prepared on a volume basis by 1:40 dilution of the 200 mg/mL solution.

Reference solutions. To analyze as the sulfate, the reference solutions must be prepared from powdered reference material in the sulfate form. The high standard is prepared in water at 6.00 mg/mL (120%) and the low standard at 4.25 mg/mL (85%) by adding 0.41 mL of water to 1.00 mL of the high standard. When the sample solution has been prepared as the free base on a volume basis, the reference solutions are prepared as the free base. Approximately 30 mg of the reference material as the sulfate is weighed and the weight converted by multiplying the weight by the ratio of the molecular weights. For example, 30.0 mg weighed as the sulfate equals 30.0 x 1163/1457 or 23.9 mg as the free base. The volume of water needed is 23.9 mg/6.00 mg/mL or 3.99 mL to prepare the 120% reference solution.

TLC analysis. The layer is developed with mobile phase (c) and dried until the absence of acetic acid odor. Zones detected using iodine-KI solution. Zones are not visible under UV light.

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Fixed Drug Combinations (FDC)

Dosage forms of some TB drugs are formulated to contain combinations of two or more components with many different ratios of the individual drugs. The methods below are for representative formulations. Samples containing Rifampin, Isoniazid, and Pyrazinamide in combination are analyzed using mobile phase (b), which separates the individual drugs adequately for quantification. If Ethambutol is present as a fourth component, mobile phase (b) can still be used because Ethambutol is not detectable under UV light and, therefore, will not interfere. Analysis of Ethambutol in the FDC requires mobile phase (a).

Isoniazid + Ethambutol (400 + 150 mg tablet)

Sample solution. The ground powder and polyethylene bag are transferred to a vessel and a volume of methanol added to produce a solution with different concentrations for each drug. This concentrated solution is diluted with different volumes of methanol to prepare the required 0.500 and 2.00 mg/mL 100% solutions. (See the methods above for the individual drugs.)

Reference solutions. Reference solutions are prepared by weighing powdered reference compound either as a primary or secondary standard. The high standard solutions are prepared as described above for the individual drugs when either an analytical balance is or is not available. A high standard is required for each drug with a concentration of 115% relative to the sample solution concentration. The low standards are prepared at a concentration of 85% relative to the sample by diluting 1.00 mL of each high standard with 0.35 mL of ethanol.

TLC analysis. Sample and reference solutions are spotted for each drug on separate sheets. The center, sample chromatogram will contain zones for both drugs on each layer. The layer is developed with mobile phase (b) for Isoniazid and (a) for Ethambutol in separate bags. Isoniazid, but not ethambutol, is detected under 254 nm UV light; both drugs can be detected by use of iodine-KI solution.

Rifampin + Isoniazid (Rifamate) (300 mg + 150 mg capsule, 150 mg + 75 mg tablet, and 150 mg + 150 mg tablet)

Sample solution. The ground powder and polyethylene bag are transferred to a vessel and a volume of methanol added to dissolve the entire contents. The volume used should be a multiple of 5 to prepare a concentrated solution having a whole-number concentration, such as 6.00 mg/mL (e.g., 300 mL is added for a tablet containing 300 mg of Rifampin). One mL of the concentrated solution is

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diluted with methanol to prepare the required 100% TLC solution. Only one solution is required for a 2:1 drug ratio. For ratios other than 2:1, the concentrated solution is diluted for each drug to prepare the two required 100% solutions. (See the methods above for the individual drugs.)

Reference solutions. The high reference standard is prepared by weighing a powder of either a primary or secondary standard. The high standard solutions (115%) are prepared by weighing exactly 7-8 mg of standard Rifampin and 4-5 mg of Isoniazid and dissolving in the correct, exact amounts of methanol to prepare the required 1.15 mg/mL and 0.575 mg/mL concentrations, respectively. The low standards (85%) are each prepared by mixing 1.00 mL of the high standards with 0.35 mL of methanol.

TLC analysis. Sample and reference solutions are spotted for each drug on separate sheets. The center, sample chromatogram will contain zones for both drugs on each layer. The layer is developed with mobile phase (b) either simultaneously or separately. Both drugs are detected under 254 nm UV light and Rifampin, less sensitively, also in visible light.

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Rifampin + Isoniazid + Pyrazinamide (300 mg + 75 mg + 400 mg tablet and 150 mg + 150 mg + 500 mg tablet)

Sample solution. For the 150 mg + 75 mg + 400 mg tablet, the ground powder and polyethylene bag are transferred to a vessel and a 50.0 mL of methanol is added to give concentrations of 3.00 mg/mL for Rifampin, 1.50 mg/mL for Isoniazid, and 8.00 mg/mL for Pyrazinamide. One mL of this solution is mixed with 2.00 mL of methanol to prepare the TLC solution for Rifampin (1.00 mg/mL) and Isoniazid (0.500 mg/mL), and 1.00 mL is mixed with 7.00 mL of methanol to prepare the Pyrazinamide solution (1.00 mg/mL). Three solutions, rather than only two, are required if the Isoniazid is in a different ratio. One mL of the concentrated solution is always diluted appropriately to prepare the individual sample solutions for drug combinations in different ratios.

Reference solutions. Directions in the previous method are followed for preparing the high standard solutions (115%) of Rifampin and Isoniazid. The required 1.15 mg/mL (115%) standard of Pyrazinamide is prepared by weighing a standard and dissolving in the correct amount of methanol. The low standards (85%) are prepared by mixing 1.00 mL of each high standard with 0.35 mL of methanol.

TLC analysis. Three separate sheets are spotted with 85% and 115% standard and sample zones. The first two sheets can be developed together in a single bag and the third sheet in a second bag. Reference tablets available: sheet 1 is spotted Rifampin + Isoniazid standards and sample and sheet 2 with Pyrazinamide standards and sample. The layers are developed with mobile phase (b)

and zones detected under 254 nm UV light. Rifampin is detected with less sensitively in visible light.

Rimampin + Isoniazid + Pyrazinamide + Ethambutol (150 mg + 100 mg + 500 mg + 267 mg)

The stated amounts are for one of several four-component FDC formulations that are available. These can be analyzed in two steps using mobile phases (a) or (b) or in one step using mobile phase (a). Rifampin is detected by visible light, Isoniazid and Pyrazinamide with UV light, and Ethambutol by iodine staining.

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