JPR INFO .com
Journal of Pharma Research

Journal of Pharma Research
Available online through
www.jprinfo.com

Review Article
ISSN: 2319-5622

An Overview of Importance of Thin Layer Chromatography on Counterfeit Drugs

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Received on: 08-12-2013; Revised and Accepted on: 27-12-2013

ABSTRACT

Counterfeit drugs are one of the key challenges facing pharmaceutical supply chains and the safety of patients. Counterfeit drug is a pharmaceutical product which is produced and sold with the intent to deceptively represent its origin, authenticity or effectiveness. It may contain inappropriate quantities of active ingredients, may be improperly processed within the body or may contain ingredients that are not on the label, and is often sold with inaccurate, incorrect, or fake packaging and labeling. Spurious drugs are a great threat to patient's life, the genuine pharmaceutical manufacturer and the image of the country as a whole. TLC is a simple, quick, and inexpensive procedure that gives the chemist a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound (preferably both run on the same TLC plate). In this paper Economical and reliable thin layer chromatography methods for rapid screening of counterfeit drugs are described. A method for rapidly screening pharmaceuticals by Thin-Layer Chromatography (TLC) has been designed for use in areas with limited resources (without electricity or in a remote area away from a laboratory) and by operators with limited training. TLC is based on the use of portable kits with standard reference tablets to eliminate weighing. Results can be reproduced by different operators and in different locations. It is especially suited for field use in developing countries. The method is low-cost, maintenance-free, fast, and reliable and also uses limited volumes of solvents. In this method, separations are performed on silica gel layers containing fluorescent indicator, and separated spots are detected under UV lamps and with iodine detection reagent. Development and iodine detection are carried out in polyethylene bags or glass jars. Sample spots are compared to reference standards developed on the same layer to identify the active ingredient and determine if its content is within the specification range.

Key words: *Thin Layer Chromatography, Rf values, Counterfeit drug, Minilab TLC, Speedy TLC.*

INTRODUCTION

1.1. Chromatography background:

At the beginning of the twentieth century, the Russian botanist Mikhail Tswett invented and named chromatography. He separated plant pigments by passing solution mixtures through a glass column packed with fine particles of calcium carbonate. The separation of those pigments appeared as colored bands on the column. Tswett named his separation method for the two Greek words "chroma" and "graphein," which mean "color" and "to write," respectively (Skoog et al., 1998). In the past six decades, chromatography has been extensively applied to all branches of science. The 1952 Nobel Prize in chemistry was awarded to A. J. P. Martin and R. L. M. Synge for their contributions to chromatographic separations, which tremendously impacted chemistry-related sciences. More impressively between 1937 and 1972, a total of 12 Nobel Prizes were based on work in which chromatography was a key tool.

In all chromatographic separations, the sample is carried by the mobile phase, which may be a gas, a liquid, or a supercritical fluid. The mobile phase is then percolated through an immiscible stationary phase that is fixed on a solid substrate. When the sample passes through the stationary phase, species are retained to varying degrees as a result of the physicochemical interaction between the sample species and the stationary phase. The separation of species appears in the form of bands or zones resulting from various retentions. Chemical information can thus be analyzed qualitatively and/or quantitatively on the basis of these separated zones. Based on the physical means by which the stationary phase and mobile phase are brought into contact.

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1.2. Introduction to TLC:

TLC is a chromatography technique used to separate mixtures (Vogel, 1989). It involves a stationary phase consisting of a thin layer of adsorbent material, usually silica gel, aluminum oxide, or cellulose immobilized onto a flat, inert carrier sheet. A liquid phase consisting of the solution is drawn up the plate via capillary action. The separation is based on the polarity of the components of the compound in question. TLC is a simple, quick, and inexpensive procedure that gives the chemist a quick answer as to how many components are in a mixture. Chromatographic principle remains the same. A small aliquot of a sample solution is applied in either a spot or band to a thin sorbent layer supported by a substrate (glass, plastic, aluminum foil) near one end of the TLC plate. After the sample has dried, the TLC plate is placed into a chamber where solvent is introduced to the end of plate where the sample was applied and capillary action wicks the solvent to the other side of the plate. Components of the sample mixture are separated-based on their different migration rates in the particular stationary and mobile phase combination. Differential migration is based on the relative affinity of each analytic for the stationary and mobile phases in the chromatographic system. Detection is often performed by visually observing the separated compounds, using either white or ultraviolet light, using necessary visualization agents to impart color or fluorescence to the compounds by using fluorogenic drivatizing agents.

TLC is employed in many areas where rapid, high-throughput, and inexpensive analysis is necessary. In the pharmaceutical field, TLC is used for identification, purity analysis, and concentration determination of active and inactive ingredients, auxiliary substances and preservatives in drug preparations, process control in synthetic manufacturing processes. Various pharmacopoeias have accepted TLC technique for the detection of impurity in a drug or chemical.

TLC is used to measure active substances and their metabolites in biological matrices in clinical and forensic chemistry. In Biochemistry, A mixture of 34 amino acids, proteins and peptides has been successfully separated and isolated from urine using silica gel plates. All these substances were found to be ninhydrin positive.

The development were carried out first with chloroform-methanol-20%ammonium hydroxide (2:2:1) and then with phenol-water.

TLC has many uses in the field of food chemistry. It is used to determine the concentration of pesticides and fungicides in water, fruits and vegetables, and meats. TLC is also used to determine the concentration of regulated substances in food, such as aflatoxins in milk products and grains and antibiotics in meats. In environmental analysis, TLC is used to measure groundwater and soil pollution. In the early days of TLC, before the advent of HPLC, researchers experimented in their laboratories with developing chambers. Various chambers were used ascending development, descending development and horizontal development (Hahn-Deinstropand Leach, 2007).

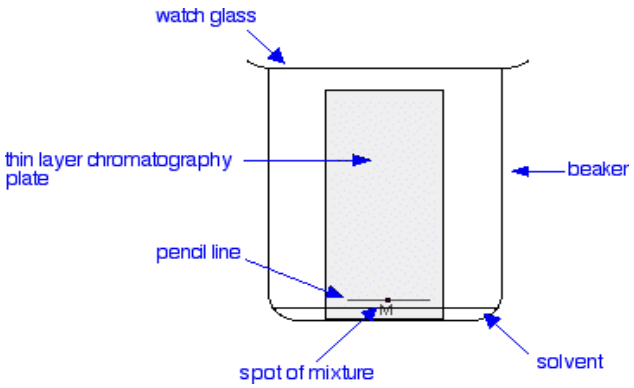


Fig. 1: Experimental setup of TLC

1.3. Definition of High-Performance Thin Layer Chromatography (HPTLC):

HPTLC is an analytical technique based on TLC, but with enhancements intended to increase the resolution of the compounds to be separated and to allow quantitative analysis of the compounds. Some of the enhancements such as the use of higher quality TLC plates with finer particle sizes in the stationary phase which allow better resolution (Reich and Schibli, 2007).

2.1. Introduction to Counterfeit drugs:

The World Health Organization estimates that up to 200,000 of the one million deaths that occur from malaria each year could be avoided if antimalarial drugs were "effective, of good quality and used correctly" (World Health Organization, 2003). In May 2008, some of the authors published a study that found 35% of antimalarial drugs sold in private shops and pharmacies in six major African cities failed basic quality control tests (Bate et al.,2008). Additionally, tuberculosis and other bacterial infections cause millions of deaths a year; drugs to combat these diseases are also routinely counterfeited (World Health Organization, 2008).

Drugs administered to patients prove their relative safety, efficacy and improved quality before they are introduced into the markets but medicines are increasingly becoming the victim of counterfeits in recent time. Counterfeit drugs are those drugs which are sold under a product name without authorization and which are sold with the intention of misleading the customer into believing that the drug is original. Counterfeiting is one of the major problems facing healthcare systems across the world. It is more prevalent in developing countries where there is limited control over the flow of drugs through the supply chain. Low-Quality Medicines poses hazards at all levels of the population and the impact of this menace can range from one section of population or escalate to full blown volcano eruption. Counterfeiters find weak links in the supply chain to introduce fake drugs and so counterfeiting market thrives in developed countries where the movement of goods in the supply chain is not strictly regulated. In the developing countries of Africa, Asia and Latin America, counterfeit drugs constitute considerable portion of the total pharmaceutical market. Counterfeiting of drugs is an economic and social menace and over the years this has grown into a well- organized criminal activity in the country. Only a sustained and concerted action backed jointly by the government, drug industry and consumer action groups can tackle it. Along with this, innovations in packaging technology have come to play a stellar role in helping the consumers identify the authentic products and to ward off counterfeiters.

Poverty, high cost of medicines, lack of an official supply chain, legislative lacunae, and easy accessibility to computerized printing technology, ineffective law enforcement machinery, and light penalties provide the counterfeiters with an enormous economic incentive without much risk. The consequences of the use of such medicines may vary from therapeutic failure to the occurrence of serious adverse events and even death.

The World Health Organization through UNICEF identified the following drugs as most essential, based upon frequency of usage and their effect on improving the quality of life:

1. Acetaminophen, all oral forms
2. Amoxicillin, all oral forms
3. Ampicillin, all oral and injectable forms
4. Benzylpenicillin (Penicillin-G), all injectable forms
5. Chloramphenicol, all oral and injectable forms
6. Chloroquine diphosphate
7. Mebendazole, all oral forms
8. Praziquantel, all oral forms
9. Quinine sulfate, all oral forms

The following drugs were added to the list to demonstrate the applicability of the method:

10. Cloxacillin, all forms
11. Estradiol cypionate
12. Sulfamethoxazole, all forms
13. Theobromine or theophylline
14. Trifluoperazine HCl

Table No. 1: Main Types of Counterfeit Drugs

Categories of Drugs	% of Total Counterfeits
Antibiotics	28
Hormones and Steroids	18
Anti-asthma and anti-allergy	8
Anti-malarial	7
Analgesics and anti-pyretics	6
Others (14 therapeutic classes)	33

2.2. Magnitude Of The Problem: [1]

The problem of spurious and counterfeit drugs has been escalating the world over in spite of tough provisions provided in drug laws in most countries to counter this problem. Between 1984 and 1999, there were 771 reports of counterfeit drugs with 78% of these coming from developing countries [2]. From January 1999 to October 2000, 46 reports of counterfeit drugs were received from [2] countries; 60% from developing countries and 40% from developed nations. The International Federation of Pharmaceutical Manufacturers Associations (IFPMA) has estimated that 7% of all drugs sold around the world are counterfeits [3].

Between January 1999 and October 2000 alone, 46 confidential reports relating to such drugs were received by WHO from [2] countries. The consumption of paracetamol cough syrup prepared with diethylene glycol (a toxic chemical used in antifreeze) led to 89 deaths in Haiti in 1995 and 30 infant deaths in India in 1998. A study conducted in WHO's South-East Asia Region in 2001 revealed that 38% of 104 antimalarial drugs on sale in pharmacies did not contain any active ingredients [4].

In India, the death penalty has been discussed as a penal action in case of conviction in a spurious drug case. 10 Fake drugs are estimated to represent 13–30% of the pharmaceutical market in India [5-7].

A survey suggested that in India's major cities one in every five medicines sold was fake [8].

According to another report released by the European Commission, 75% of global cases of counterfeit medicines originated from India [9].

India is also the major exporter of counterfeit drugs to the least developed countries such as Nigeria, including anti-HIV drugs [10].

The human right to a standard of living adequate for health and well-being is an important right that is recognised in the International Bill of Human Rights [11].

Although the Indian Constitution does not explicitly mention health or health care as a fundamental right, the legal right to health is based on right to life and liberty (article 21 in part III of the Constitution of India). This right to health has to include the right to access quality medicines to be of any value; thus, the state is duty bound to provide quality medicines to its people.

2.3. Types of Counterfeit Drugs and their consequences:

Illegal drugs are often produced and sold with the intent to deceptively represent their origin, authenticity or effectiveness. The nature of these fraudulent drugs ranges from those containing no active ingredient (eg. when a bag of powdered lactose claimed to be cocaine), with insufficient active ingredient or with some diluents (e.g., Baking soda or lactose) or sometimes with a wrong active ingredient (e.g., when methamphetamine is sold as cocaine) or with a fake packaging [12-13]. The various types of counterfeit drugs are:

1. Counterfeit drugs containing same dose of the active ingredient,
2. Mislabeled medications,
3. Counterfeit drugs containing an incorrect dose of the active ingredient,
4. Counterfeit drugs which do not contain the active ingredient,
5. Counterfeit drugs containing a potentially harmful substance,
6. Counterfeit drugs containing an unlisted active ingredient.

2.4. Factors Encouraging counterfeiting of Drugs: [14]

A variety of factors account for why medicines are attractive for counterfeiting. Medicines are high value items in relation to their bulk and the demand for medicines is infinite. Furthermore, for the counterfeiter, ingredient costs can be very low if cheap substitutes are used or if these are omitted altogether, as is often the case. Producing counterfeit drugs may not require building huge infrastructure or facilities. They can be produced in small cottage industries or in backyards or under the shade of a tree. There are also no overhead costs due to quality assurance or meeting Good Manufacturing Practices (GMP) standards, since such standards are never implemented and gross margins are therefore very high.

A counterfeit drug has a better capacity to deceive, particularly if it is copied to make it look like the original product and if it comes from a supposedly legitimate source so that purchasers are unlikely to be suspicious. Moreover, the process by which patients get their drugs is different from that for other consumer goods: doctors or health workers prescribe them. Even when patients choose their own drugs they may lack the specialized knowledge to detect whether the product they are buying is of good quality let alone be able to detect whether the product is counterfeit. Other factors that encourage counterfeiting of medicines are discussed below.

Guidelines for the development of measures to combat counterfeit drugs.1999 WHO;Geneva, Switzerland.

1. Lack of political will and commitment
2. Lack of appropriate drug legislation
3. Absence of or weak drug regulation
4. Absence of adequately staffed or technically equipped to monitor
5. Weak enforcement and penal sanctions
6. Corruption and conflict of interest
7. Demand exceeding supply
8. High prices of medicines
9. Inefficient cooperation between stakeholders
10. Lack of regulation by exporting countries and within free trade zones
11. Trade through several intermediaries

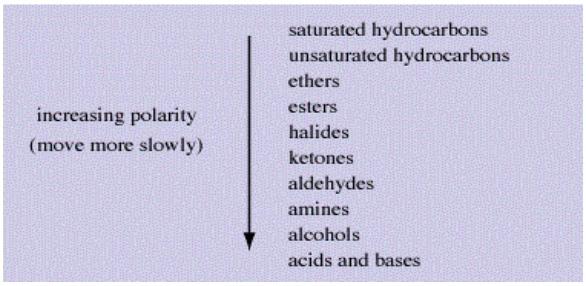
3.1. The Solvents Choice in TLC:

When you need to determine the best solvent or mixture of solvents (a "solvent system") to develop a TLC plate or chromatography column loaded with an unknown mixture, vary the polarity of the solvent in several trial runs: a process of trial and error. Carefully observe and record the results of the chromatography in each solvent system. You will find that as you increase the polarity of the solvent system, all the components of the mixture move faster (and vice versa with lowering the polarity). The ideal solvent system is simply the system that gives the best separation.

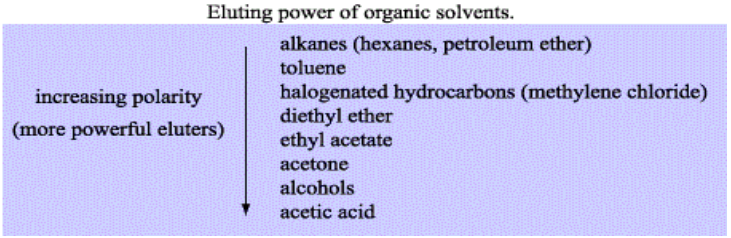
TLC elution patterns usually carry over to column chromatography elution patterns. Since TLC is a much faster procedure than column chromatography, TLC is often used to determine the best solvent system for column chromatography. For instance, in determining the solvent system for a flash chromatography procedure, the ideal system is the one that moves the desired component of the mixture to a TLC R_f of 0.25-0.35 and will separate this component from its nearest neighbor by difference in TLC R_f values of at least 0.20. Therefore a mixture is analyzed by TLC to determine the ideal solvent(s) for a flash chromatography procedure.

Beginners often do not know where to start: What solvents should they pull off the shelf to use to elute a TLC plate? Because of toxicity, cost, and flammability concerns, the common solvents are hexanes (or petroleum ethers/ligroin) and ethyl acetate (an ester). Diethyl ether can be used, but it is very flammable and volatile. Alcohols (methanol, ethanol) can be used. Acetic acid (a carboxylic acid) can be used, usually as a small percentage component of the system, since it is corrosive, non-volatile, very polar, and has irritating vapors. Acetone (a ketone) can be used. Methylene chloride or and chloroform (halogenated hydrocarbons) are good solvents, but are toxic and should be avoided whenever possible. If two solvents are equal in performance and toxicity, the more volatile solvent is preferred in chromatography because it will be easier to remove from the desired compound after isolation from a column chromatography procedure. Ask the lab instructor what solvents are available and advisable. Then, mix a non-polar solvent (hexanes, a mixture of 6-carbon alkanes) with a polar solvent (ethyl acetate or acetone) in varying percent combinations to make solvent systems of greater and lesser polarity. The charts below should help you in your solvent selection.

The expected elution order of organic classes.



Eluting power of organic solvents.



3.2. Interactions between the Compound and the Adsorbent:

The strength with which an organic compound binds to an adsorbent depends on the strength of the following types of interactions: ion-dipole, dipole-dipole, hydrogen bonding, dipole induced dipole, and van der Waals forces. With silica gel, the dominant interactive forces between the adsorbent and the materials to be separated are of the dipole-dipole type. Highly polar molecules interact fairly strongly with the polar SiOH groups at the surface of these adsorbents, and will tend to stick or adsorb onto the fine particles of the adsorbent while weakly polar molecules are held less tightly. Weakly polar molecules generally tend to move

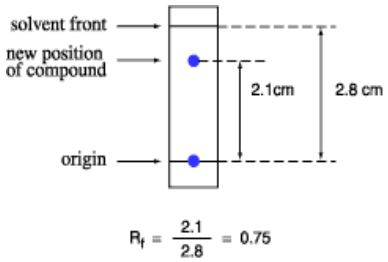
through the adsorbent more rapidly than the polar species. Roughly, the compounds follow the elution order given above.

The R_f value:

The retention factor, or R_f, is defined as the distance traveled by the compound divided by the distance traveled by the solvent.

$$R_f = \frac{\text{distance traveled by the compound}}{\text{distance traveled by the solvent front}}$$

For example, if a compound travels 2.1 cm and the solvent front travels 2.8 cm, the R_f is 0.75:



The R_f for a compound is a constant from one experiment to the next only if the chromatography conditions below are also constant:

- Nature and thickness of the adsorbent: Different adsorbents will give different Rf value for same solvent. Reproducibility is only possible for given adsorbent of constant particle size and binder.
- Solvent system:The purity of solvents and quantity of solvent mixed should be strictly controlled.
- Temperature: As the temperature is increased, Volatile solvents evaporate more quickly, solvents run faster, and Rf values generally decrease slightly.
- Amount of material spotted: Increasing the mass of sample on the plate will often increase the Rf of drug, especially if it normally tails in the system.
- Thickness of layer: Standard plates approximately 250 micrometer is the preferable thickness of layer.

Since these factors are difficult to keep constant from experiment to experiment, relative R_f values are generally considered. "Relative R_f" means that the values are reported relative to a standard, or it means that you compare the R_f values of compounds run on the same plate at the same time. The larger an R_f of a compound, the larger the distance it travels on the TLC plate. When comparing two different compounds run under identical chromatography conditions, the compound with the larger R_f is less polar because it interacts less strongly with the polar adsorbent on the TLC plate. Conversely, if you know the structures of the compounds in a mixture, you can predict that a compound of low polarity will have a larger R_f value than a polar compound run on the same plate.

The R_f can provide corroborative evidence as to the identity of a compound. If the identity of a compound is suspected but not yet proven, an authentic sample of the compound, or standard, is spotted and run on a TLC plate side by side (or on top of each other) with the compound in question. If two substances have the same R_f value, they are likely (but not necessarily) the same compound. If they have different R_f values, they are definitely different compounds. This identity check must be performed on a single plate, because it is difficult to duplicate all the factors which influence R_f exactly from experiment to experiment.

3.3. TLC Detection:

The presence of a fluorescent indicator is necessary for the detection of drugs that quench fluorescence under 254 nm UV light. Blue fluorescent quenched spots may be viewed on a bright green background under 254 nm ultraviolet (UV) light and of brown spots in white light after dipping plates in iodine-KI solution or exposing the plates to an iodine vapor mist. The staining of a Thin Layer Plate with iodine vapor is among the oldest methods for the visualization of organic compounds. It is based upon the observation that a iodine vapor has a high affinity for both unsaturated and aromatic compounds.

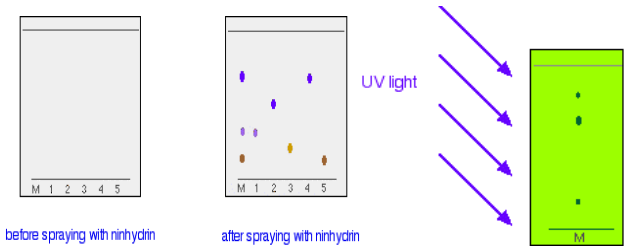


Fig. 2: Procedure of Detection of TLC Plate

3.4. Troubleshooting of TLC:

Examples of common problems encountered in TLC:

- The compound runs as a streak rather than a spot: The sample was overloaded. Run the TLC again after diluting your sample. Or, your sample might just contain many components, creating many spots which run together and appear as a streak. Perhaps, the experiment did not go as well as expected.
- The sample runs as a smear or a upward crescent: Compounds which possess strongly acidic or basic groups (amines or carboxylic acids) sometimes show up on a TLC plate with this behavior. Add a few drops of ammonium hydroxide (amines) or acetic acid (carboxylic acids) to the eluting solvent to obtain clearer plates.
- The sample runs as a downward crescent: Likely, the adsorbent was disturbed during the spotting, causing the crescent shape.
- The plate solvent front runs crookedly: Either the adsorbent has flaked off the sides of the plate or the sides of the plate are touching the sides of the container (or the paper used to saturate the container) as the plate develops. Crooked plates make it harder to measure R_f values accurately.
- Many random spots are seen on the plate: Make sure that you do not accidentally drop any organic compound on the plate. If get a TLC plate and leave it laying on your workbench as you do the experiment, you might drop or splash an organic compound on the plate.
- If blur of blue spots on the plate as it develops: Perhaps you used an ink pen instead of a pencil to mark the origin?
- No spots are seen on the plate: It is because of the solution of the compound is too dilute. Concentrating the solution, or spot it several times in one place, allowing the solvent to dry between applications. Some compounds do not show up under UV light; we have to try another method of visualizing the plate (such as staining or exposing to iodine vapor). Or, perhaps you do not have any compound because your experiment did not go as well as planned. If the solvent level in the developing jar is deeper than the origin (spotting line) of the TLC plate, the solvent will dissolve the compounds into the solvent reservoir instead of allowing them to move up the plate by capillary action. Thus, you will not see spots after the plate is developed. These photos show how the yellow compound is running into the solvent when lifted from the developing jar.

3.5. Procedures of Detection of Counterfeit Drugs:

The detection and prosecution of criminals who market counterfeit pharmaceuticals have several stages. First of all, suspect products have to be traced. The drug, sampled according to an

established procedure, should undergo defined physical or organoleptic examination by the drug inspector. If the results indicate that the drug formulation may be a counterfeit product, then at least some chemical tests must be repeated to confirm the necessity for further analysis. Drugs are then analysed by simple tests, including TLC. If these tests do not provide conclusive evidence and the drug is still considered to be a possible counterfeit, then a compendia procedure is required.

Throughout the investigation, it is assumed that a chain of custody has been established, i.e. the correct procedures were followed before the drug was received for analysis by the laboratory. This ensures that the results of these examinations are reliable and will be accepted as valid in future steps, e.g. prosecution of the supplier of the counterfeit pharmaceuticals. The final results shall be submitted to the appropriate official in the drug regulatory authority.

First Step:

Visual inspection: Irrespective of the analytical method used, the first step in identifying potential counterfeit drugs is the careful visual inspection of the product, and its packaging and labeling. A comparison with the authentic drug product is always preferred. Differences in labeling, packaging and the physical appearance of dosage form, e.g. shape, colour, etc., indicate a potential counterfeit [15-17].

Many fake medicines have been found at this step, but in some cases they are becoming harder to spot in this way because of an improved quality of copying the genuine packaging in the manufacturing process. For example, holograms placed on packages of antimalarial tablets since 1996 to thwart counterfeiters are now being reproduced more faithfully and are much harder to distinguish from fakes [18].

Even in the absence of knowledge of the physical characteristics of the authentic drug, a visual inspection may indicate that there has been tampering, that there is non-uniform coloration of the drug product under investigation, etc. Again such observations signal the possibility of a counterfeit.

Legitimate drug manufacturers should be encouraged to collaborate with national DRAs and with WHO by providing information and materials on the physical attributes of their products; this would be also be to their own benefit.

Second Step:

Disintegration: A dissolution and disintegration test is then carried out by dropping a tablet or capsule in warm (37^0C) water contained in a 100 mL wide neck bottle and swirling periodically. Unless the product is labeled “slow relea-se” or “enteric”, it should disintegrate within 30 min, measured with a pre-set timer, or be suspected of being illegal.

Third Step:

Color reaction & TLC: The third stage is the use of simplified test tube color reactions for a quick check of the presence of any amount of a drug active ingredient in the sample. In this case, a yes/no response is not adequate, and the method must be at least semi quantitative, like TLC.

3.6. Successful implementation of simple tests:

The following points should be taken into consideration:

- The costs of performing simple and other tests for the detection of counterfeits should be weighed against the larger costs of drug injury, ineffective therapy and possible patient deaths.
- Guidelines for official organoleptic detection procedures should be widely available to all relevant persons. Pharmaceutical manufacturers should be encouraged to collaborate with national DRAs in the provision of information and appropriate materials dealing with the physical attributes of their products.
- All available technical documents should be translated into the official/national language(s).
- Consideration should be given to the application of rapid quantification procedures when counterfeit products have been positively identified.
- The type of systems to be used should be carefully considered before anyone is selected for training in counterfeit testing. Some methods, e.g. high-performance TLC, are sophisticated and have proved too difficult for less qualified personnel in previous training programmes.

3.7. Developing training programmes: inspection and examination of counterfeit pharmaceuticals:

The following guidelines are a modified version of the provisional guidelines for developing training programmes approved by the Thirty-fifth Expert Committee on Specifications for Pharmaceutical Products [19], and subsequently considered by the International Workshop on Counterfeit Drugs [20].

3.8. Complementary Analytical Methods:

Where sophisticated counterfeits are present, testing will require the use of advanced analytical techniques such as mass spectrometry, nuclear magnetic resonance, etc. High-technology techniques, such as those using a near-infrared spectrophotometer, are also useful. The apparatus is simple to operate and can be used for the identification and semi-quantification of active ingredients in dosage forms. It is available as a portable unit requiring a very small amount of sample and little sample preparation, and gives results in a matter of minutes with the help of computerized controls. While the initial cost of such technologies may be an inhibiting factor, this should be weighed against the advantages they provide in terms of quick and accurate detection of counterfeit drugs. It should also be considered against the costs of training personnel in other methods and of acquiring and maintaining the supplies of reagents and other special materials required for those methods [21].

3.9. Methods based on thin-layer chromatography:

TLC screening procedures are recommended for the detection of counterfeit drugs. Numerous studies have demonstrated the multiple uses of these methods. They can be employed for the identification of drug substances, the estimation of drug substance content and the detection of related substances which could be regarded as impurities. TLC procedures are more specific and selective than WHO basic tests for the identification of drug substances and are also subject to less interference by excipients.

A counterfeit product may contain the correct active ingredients but in amounts other than those declared. In response to effective anti-counterfeit measures, counterfeiters have often introduced small quantities of the genuine pharmaceutically active substances into the dosage forms. This gives positive identification results and in this way counterfeiters attempt to foil or confound the process of detection. In such cases, the basic tests are inadequate; TLC procedures are therefore preferred, as they are capable of giving semi-quantitative information on the active ingredient and also on any related substances in the dosage forms.

4.1. TLC Methods: [22]

A previously developed TLC method [23] was useful for rapidly screening drugs by comparing the sample solution with different concentrations of reference material. The success of their system required the laboratory to have electricity and special equipment. Even though their method was fast and economical, and the operator required little training, it did not meet the needs of locations without electricity and with only limited resources. This work was [24] directed toward the development of a rapid screening system which could be performed either in a well-equipped laboratory or in remote areas with or without electricity, and by persons having limited technical background.

According to World Health Organization (WHO):

"A counterfeit medicine3 is one which is deliberately and fraudulently mislabeled with respect to identity and/or source”. They include products with a different drug but none of the labeled active ingredient, the correct active ingredient at the wrong level, or the correct drug and amounts in the wrong packaging. Thin layer chromatography (TLC) is the main screening method used today to decide if a drug product meets label specifications and is legal. Drug screening TLC methods are simple, quick, inexpensive, selective, and semi quantitative and they can be used in the laboratory or open air and in locations such as a port of entry, distribution center, clinic, pharmacy, or hospital. No electronic measurements are needed, and estimation is made from visual inspections under daylight. TLC can give an indication whether the active ingredient is present and its level of content, and, therefore, if the product is qualified or authorized or legal on this basis. Some related substances may also be detected and quantified. However, TLC will not detect counterfeits that have wrong active or inactive ingredients if they are not visualized by the detection method being used for the correct active drug. Chemicals must be handled properly, and all

analyses must be performed in areas with ventilation, preferably in a hood with a suitable air flow if one is available.

4.2. Reagents & Solvents:

The number of chemicals used as solvents and developers must be minimal if a method is to be successful in areas with limited supplies. Table 2 shows the minimum number of chemicals needed for the analyses of the above drugs. This list is not necessarily complete, but it is sufficient to begin such analyses. Chloroform has been used as a developer solvent even though it is carcinogenic; it was used in our well-equipped laboratory where suitable handling facilities were available. All TLC can be performed with other development solvents so chloroform can be eliminated. Studies are under way to find other solvents by using the polar series of chemicals to establish a system free of chloroform.

Table No. 2: Common Chemicals needed for TLC analyses

Acetone (4 L)	Ethyl acetate (4 L)	Iodine crystals (100 g)
Ammonium hydroxide (0.5L)	Formic acid (0.5L)	Methanol (4 L)
Chloroform (4 L)	Glacial acetic acid (0.5L)	Potassium iodide (100 g)
Ethanol, 95%	Hydrochloric acid (0.5L)	Toluene (4 L) & Distilled water

All solvents have been found compatible with the polyethylene bag. The volume of solvents required was kept at a minimum to reduce cost, decrease exposure to chemicals, and decrease waste disposal. It was also found that measuring small volumes accurately is difficult with pipets or limited equipment. Experience showed that pipets are impractical for use by the unskilled analyst, whereas graduated syringes are easy to use. Although the graduated syringe is not as accurate as volumetric glassware, it has sufficient accuracy for this type of estimation.

However, because chloroform is carcinogenic, it may be desirable to substitute another solvent from the polar series. Any developing system may be used as long as the relative retention lies between 0.1 and 0.8. The ability to analyze these drugs visually in white light due to a change in intensity of the spots with concentration was verified by measuring the intensity in the UV at 254 nm with a densitometer.

Chloroform has been used as a developer solvent even though it is carcinogenic; it was used in our well-equipped laboratory where suitable handling facilities were available. All TLC can be performed with other development solvents so chloroform can be eliminated. Studies are under way to find other solvents by using the polar series of chemicals to establish a system free of chloroform.

The chemicals listed here are the most widely used for TLC analyses; however, additional solvents may be necessary for other pharmaceuticals.. As emphasized above, the rapid screening of pharmaceuticals is intended to be used in areas where equipment and training are limited. If the analyses were performed in well-equipped laboratories with highly trained personnel, it would only be necessary to indicate the final concentration needed.

The drugs were used to establish the suitability of the apparatus for rapid screening apparatus of pharmaceuticals. All analyses were performed either with USP primary standards or with secondary standards which had been compared previously to the primary standards.

The formulated drugs in normal dosage forms and contents were obtained from commercial pharmaceutical suppliers. The concentrations needed for the sample and standards were prepared by weighing and diluting aliquots.

The specifications for a single dosage unit call for the drug content to fall between 85 and 115% of the declared content for most drugs and between 85 and 120% for the antibiotics. This criterion was used to establish the suitable conditions for the reference solutions. The drug contents of the reference tablets were determined on the basis that a single tablet contained the quantity necessary to prepare a concentration equivalent to the highest allowable concentration of the sample (115 or 120%).

5.1. The analysis of counterfeit Drugs:

The ability to analyze these drugs visually in white light due to a change in intensity of the spots with concentration was verified by measuring the intensity in the UV at 254 nm with a densitometer. Plots of concentration versus intensity were found to be linear with a correlation of 0.99+ for all drugs tested. The densitometer measurements demonstrated that differences in intensity were sufficient for visual analysis. Because spots vary in size with concentration, size and intensity differences can readily be detected visually. All TLC sheets were dipped into a solution of iodine after the UV measurements, and the intensities were compared visually. Again the differences could be seen well enough to decide whether the drug was within specifications. The results showed that if reference tablets were available, drugs could be rapidly screened with the same confidence as a comparison with USP standards. The data established the quantity of drug required in each reference tablet and the conditions for analysis. To be suitable, the reference tablets must be stable over a period of time and variation in temperature. In many areas of the world, daily temperatures range around 40°C during a large part of the year. All the drugs listed in Table 1 were tested for stability over a period of 1 year at 40°C under anhydrous conditions. Since no reference tablets existed at this stage of the investigation, formulated drugs in normal dosage forms were used. The formulated drugs were stored in sealed glass bottles and in a 40°C oven. Samples were removed at intervals and analyzed by liquid chromatography using high and low concentrations methods to detect possible degradation and assay. USP primary standards were used as references [25]. The listed drugs showed no degradation when not exposed to moisture at this elevated temperature. Some drugs were in capsules and others were in tablet form. It would be expected that drugs would be more stable in tablet form than powder. To test the concept of using reference tablets in rapid screening of drugs by TLC, the following 3 drugs were selected from the essential drug list: acetaminophen, ampicillin, and chloroquine phosphate. These drugs were selected because of the broad range of differences in concentration needed for suitable visual analysis. The reference tablets were prepared by the Department of Pharmacy, University of Maryland. If reference tablets of drugs were available, neither the sample nor reference would need to be weighed, and the complete analysis could be done in remote areas or away from a laboratory.

The total weight of each of the reference tablets was selected to be 100 mg for convenient handling. This meant that different reference tablets would contain a wide range of excipients. The excipient content ranged from slightly over 50% to 97+%. Table 6 shows the suggested weight for the active drug, the measured assay, the standard deviation, and the percent of the expected assay. The measured content of the active drug and standard deviation would be supplied. The volume of solvent needed for the high standard is determined by dividing the weight (mg) by the concentration needed for the high standard (mg/mL). The volume can be measured by a 5 mL graduated syringe which is accurate to within the overall accuracy of the analysis. All reference tablets were formulated to disintegrate quickly in the solvent system to eliminate grinding. The assays listed for the tablets were determined by liquid chromatography with (paracetamol) with the USP standards and the reference tablets.

The linearity of the spot intensity as a function of concentration was checked by densitometer in the UV at 254 nm. It was possible in some cases to measure the intensity of the iodine spots in the visible range, but in most cases it was difficult because the spots and background changed with time. Correlations of the UV intensities by least squares fit of intensity versus concentration for the 3 drugs were in the range of 0.99+ for the reference tablets and USP standards.

5.2. Minilab TLC System: [26]

The Minilab TLC analysis identifies the active ingredient by compari-son of distance of travel (R_F value) between the sample spot and an authen-tic standard spotted on the same plate, and semiquantitative proof of con-tent is made by visually comparing the color, size, and intensity between the sample spot and reference spots for each method of detection. Every drug has a detailed individual monograph for its analysis. As an example, the monograph for cotrimoxazole has the following sections: principle, equipment and reagents, preparation of the stock standard solution from the reference tablet, preparation of the 100% working standard solution (upper working limit), preparation of the 80% working standard solution (lower working limit), preparation of stock standard solution from a tablet claiming a potency of 120 or

240 mg cotrimoxazole per unit, preparation of the working sample solution, spotting, development (including the mo-bile phase composition and development time), detection, example of the chromatoplate observed at 254 nm (Fig. 4), observations to be made at 254 nm, observations to be made during iodine staining, and results and actions to be taken. Some drug monographs include a third detection method, e.g., anisaldehyde solution for artesunate.

A proficiency test was carried out recently to assess the performan-ce of Minilab visual TLC quantification estimates [27]. Samples were made at 0, 40, and 100% from a drug reference tablet and given, unidentified, to inspectors with the Minilab protocol for quality screening. In round 1 of the proficiency test, only three of 28 substandard samples were correctly iden-tified. Round 2, administered after a performance qualification test for the analytical method, showed improvement: 19 of 27 substandard drugs were correctly identified, while five out of nine inspectors made the correct infe-rence on the quality of 45 samples. In both rounds, two inspectors failed to identify substandard samples. These results show the need to have com-petent, well trained users and to include a proficiency test in the Minilab screening program in order to obtain reliable results.

CONCLUSION

TLC screening procedures are recommended for the detection of counterfeit drugs. Numerous studies have demonstrated the multiple uses of these methods. They can be employed for the identification of drug substances, the estimation of drug substance content and the detection of related substances which could be regarded as impurities. TLC procedures are more specific and selective than WHO basic tests for the identification of drug substances and are also subject to less interference by excipients.

A counterfeit product may contain the correct active ingredients but in amounts other than those declared. In response to effective anticounterfeit measures, counterfeiters have often introduced small quantities of the genuine pharmaceutically active substances into the dosage forms. This gives positive identification results and in this way counterfeiters attempt to foil or confound the process of detection. In such cases, the basic tests are inadequate; TLC procedures are therefore preferred, as they are capable of giving semi-quantitative information on the active ingredient and also on any related substances in the dosage forms.

Portable labs that perform thin-layer chromatography (TLC) provide a relatively inexpensive, versatile, and robust means of identifying substandard drugs at a fraction of the resources required for modern laboratory testing.

One critical advantage of TLC is that it is established within the academic literature, which means its results are more accepted by government agencies.

Overall, choice of technology will come down to a variety of factors: how quickly results are required (spectrometry is generally quicker; however given that different but bioequivalent products produce different spectra, methods must be established for all new brands, which means the initial setup time can be longer for spectrometers); cost (TLC is less expensive – at most $10,000 for a fully equipped lab and training costs for one person, compared with approximately $50,000 for a spectrometer and training); reliability of results to an uninitiated user

ACKNOWLEDGEMENT

We would like to express our sincere gratitude to Mr. C. Hariharan, Director, Central Drugs Laboratory and Mrs. Suparna Maitra, Incharge of Pharma Research Section, Central Drugs Laboratory, Kolkata and Mrs. J. Xalco, Incharge of Training Section, Central Drugs Laboratory, Kolkata and other Staffs of Central Drugs Laboratory have been source of constant inspiration to us.

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Conflict of interest: The authors have declared that no conflict of interest exists.

Source of support: Nil