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ANALYSIS OF COUNTERFEIT DRUGS BY THIN LAYER CHROMATOGRAPHY

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SUMMARY

Economical and reliable thin layer chromatography methods for rapid screening of counterfeit drugs are described. The methods, which can be carried out in the field by inspectors with limited expertise, are based on the use of portable kits with standard reference tablets to eliminate weighing. Separations are performed on silica gel layers containing fluorescent indicator, and separated spots are detected under ultraviolet 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. Additional methods described are visual inspection of packages and their contents and simple colorimetric tests for active ingredients, as well as column chromatographic and spectrometric methods for augmenting and validating TLC results.

INTRODUCTION

Counterfeit drug products and active pharmaceutical ingredients are a great concern to government regulatory agencies, pharmaceutical companies, health care providers, and consumers [1,2]. Newton et al. [3] reported that the production of counterfeit or substandard drugs is a widespread and under-recognized problem that contributes to morbidity, mortality, and drug resistance, and leads to spurious reporting of resistance and toxicity and loss of confidence in health care systems; people in poor countries are most disadvantaged; and there is very little research and information on the counterfeits considering the great size of the problem. Mukhopadhyay [4] cited specific cases of the seizure in the United Kingdom of 5,000 counterfeit packets of the flu medication Tamiflu worth close to $1,000,000 in 2006; the deaths of 2,500 people in Niger in 1995 upon using fake donated vaccines to fight a menengitis epidemic; the deaths of 30 infants in India in 1998

after they were given cough syrup that contained diethylene glycol; and the deaths of at least 30 people in Cambodia in 1999 after taking older and less effective antimalarias that were packaged and sold as the more potent and expensive drug artesunate. Additional deaths of children occurred in Haiti and Nigeria due to ethylene glycol scams [5]. A multinational survey of fake antimalarias in Southeast Asia (Burma, Lao PDR, Vietnam, Cambodia, and Thailand), published in 2004 [6], found that none of the 188 tablet packs labeled "artesunate 53%" that were purchased and tested contained any artesunate, and 9% of the 44 mefloquin samples analyzed contained <10% of the expected amount of the active ingredient. The World Health Organization (WHO) has estimated that 25% of medicines in developing countries are counterfeit, with a figure as high as 50% in some places, and that malaria, tuberculosis (TB), and HIV/AIDS drugs are most counterfeited; the U.S. Center for Medicine in the Public Interest has predicted that global counterfeit drug sales will reach $75,000,000,000 in 2010, a 95% increase since 2005 [4]. All countries, regardless of efforts in drug regulation, are affected by this increase, especially in light of the ease of purchase of questionable drugs on the internet; however, developing countries are at greatest risk, so the costs of analytical screening methods are critically important.

TLC METHODS

According to the WHO, counterfeit drugs are defined as mislabeled medicines manufactured with substandard safety, quality, and effectiveness. 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, inexpensive, selective, and semiquantitative, and they can be used in the laboratory or in the field in locations such as a port of entry, distribution center, clinic, pharmacy, or hospital. 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.

Speedy TLC Kit

In a series of papers starting in 1989, A.S. Kenyon, T.P Layloff, and coworkers developed simple and inexpensive TLC methods for rapid screening of counterfeit drugs that can be used either in a well equipped laboratory or in remote areas with or without electricity and by personnel with limited technical background and training. Balances are not necessary if standard tablets of the drugs are available, the procedure can be performed safely in the open air with no hood, and estimates are made from visual inspections in daylight without electronic measurements.

The first paper in the series reported a method for estimating the quality of theophylline tablets prescribed to treat respiratory disease [7]. Aluminum-backed silica gel F_{254} layers (5 x 10 cm) were developed with chloroform-acetone (1:1) mobile phase, after which the dried layer was dipped into a iodine-KI solution to make the theophylline spots visible. Three solutions were spotted for visual comparison: the standard tablet, the sample tablet, and diluted sample tablet at the low end of the acceptable content range. The dissolving of standard and sample tablets, mobile phase development, and detection were carried out in polyethylene bags; the development bag was held in a plate support cut from 0.3 mm thick aluminum.

A more general variation of this method was reported [8] for 10 pharmaceuticals (ampicillin, benzylpenicillin, chloramphenicol, chloroquine diphosphate, estradiol cypionate, paracetamol, praziquantel, sulfamethoxazole, theophylline, and trifluoperazone HCl). Glass-backed 5 x 10 cm silica gel 60 F_{254} plates (E. Merck, Darmstadt, Germany), development with appropriate mobile phases in standard TLC containers, and detection of black fluorescence-quenched spots on a bright green background under 254 nm ultraviolet (UV) light and of brown spots in white light after dipping the plate in iodine-KI solution contained in a plastic bag were employed. Visual analysis was carried out by spotting the sample between standards bracketing a range above and below the declared drug content of each medicine, and densitometry at 254 nm before iodine detection and at 420 nm after was used to confirm that valid semiquantitative analysis could be attained visually.

The rapid visual TLC screening method was next expanded to determine 13 drugs, including three on the WHO Essential Drug List [9]. The development bag was now supported in an apparatus known as "Speedy TLC", available from Granite Engineering, Inc. (Granite City, IL USA).

A simple, at-site detection of diethylene glycol/ethylene glycol con-

tamination of glycerin and glycerin-based raw materials was described using Merck silica gel coated 5 x 10 cm plastic sheets; toluene-acetone-5 M ammonium hydroxide, 5:85:10, mobile phase with development in the Speedy TLC apparatus; and visual detection directly as dark spots on a light background or after staining with iodine-starch or potassium permanganate reagent. The method cost less than $1.00 per test and required 20 min [10].

In two studies [11,12], a substantial number of substandard TB drugs were found in samples collected in Colombia, Estonia, India, Latvia, Russia, Vietnam, and other countries using the rapid TLC screening method in a laboratory setting. The specific procedures for the application of this system for the rapid screening of TB pharmaceuticals, which have been used successfully in underdeveloped countries in the field, were then described in detail [13]. In this report, the Speedy TLC apparatus was used for screening a single content of particular TB pharmaceuticals at a given concentration. Two reference solutions representing the upper and lower dosage limits (85% and 115 or 120% of the correct value, depending on the legal specification of the drug being analyzed) are spotted on the plate, 2 cm up from the bottom edge, in 3.0 μL aliquots with a sample solution representing 100% spotted between the standards. After development, the spots are examined visually under UV light and in visible light after detection by KI-iodine solution.

No analytical balance is required for sample or standard solution preparation. A sample tablet is ground to a fine powder in a small polyethylene bag, and the bag and powder are transferred to a suitable vessel (e.g., a beaker, flask, or bottle); the contents of capsules are simply emptied into a vessel. The proper volume of solvent is added, and the vessel is shaken vigorously to dissolve the powder in order to prepare a concentrated solution from which the TLC sample solution is prepared by dilution.

The high reference solution, equivalent to 115 or 120% when the sample is prepared to be 100%, is prepared by dissolving a reference standard tablet containing a predetermined quantity of the drug in a fixed volume of solvent; reference tablets are formulated to dissolve completely in the solvent without grinding. The low reference solution (85.0% relative to the sample) is prepared by dilution of the high reference solution.

TLC analysis is performed using the Speedy TLC kit supplied with plastic bags, holders, and all other required accessories. Merck plastic-backed silica gel 60 F_{254} 5 cm · 10 cm sheets are required for use in the kit apparatus; the presence of fluorescent indicator in the layer is necessary for detection of drugs that quench fluorescence under 254 nm UV light. The

specified mobile phases will provide the required separation for each analysis with drug R_F values between 0.2 and 0.8.

The rigid aluminum TLC frame, 10 cm plastic bag, filter paper saturator strips, aluminum developing tray, and clamp and fishhook are assembled, and the mobile phase is added. The TLC 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 mobile phase 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 15 to 18 mL of the mobile phase specified for the particular drug being analyzed. The development bag and back-to-back aluminum trays will accommodate two sheets at a time.

The spots for all drugs can be detected, after drying the layer, under 245 nm UV in a TLC viewing box or in an unlighted room, and/or by iodine staining. The KI-iodine staining solution is placed in a plastic bag, the sheets are immersed in the reagent, and they are then removed and the spots observed after excess reagent is evaporated. Most drugs are detectable using the iodine reagent, so this method is applicable when UV light is unavailable (e.g., absence of electricity or batteries to power a UV lamp).

After detection, visual inspection is made to assure that the sample spot size and intensity are between those of the standards. Other criteria for an acceptable drug analysis include no additional, unexplained spots in the sample and exact line up of standard and sample spots (identical R_F values). If the sample spot appears to be at or near the lower limit or outside of the range of the high and low standard spots, confirmation is obtained by another method (see below). The recipe for the KI-iodine reagent and the preparation and use of the plastic bags are described in detail in the original paper [13], along with three mobile phase compositions and drug R_F values. Experimental details are given in the paper for standard and sample preparation and TLC analysis of ethambutol hydrochloride (100 and 400 mg tablets), isoniazid (100 and 300 mg tablets), pyrazinamide (400 mg tablets), rifampin (150 md capsules), streptomycin sulfate (200 mg mL^{-1} injectable), and various fixed combinations of two or three of these drugs.

Fast Chemical Identification System (FCIS)

The FCIS was developed in China comprising two color reactions based on functional groups in the molecules and two TLC methods for screening of fake macrolide antibiotics. Sulfuric acid as a common reaction of macrolides is first used to distinguish them from other types of drugs, then 14- and 16-membered macrolides are classified by potassium permanganate reactions depending on the time for loss of color in the test solution. Two TLC analyses on silica gel GF_{254} plates are used for further identification; the mobile phase is ethyl acetate-hexane-conc. ammonium hydroxide, 100:15:15, for 14-membered macrolides and trichloromethane-methanol-conc. ammonium hydroxide, 100:5:1, for 16-membered macrolides. A suspected counterfeit macrolide preparation can be identified within 40 min [14].

MINILAB TLC SYSTEM

The main field screening method in use today is based on the portable Minilab kit (Fig. 1), developed by the German Pharma Health Fund

Fig. 1

Minilab drug screening kit (photograph supplied by GPHF)

(GPHF) [5] after earlier studies of the analysis of two series of essential drugs using easily accessible TLC materials [15,16]. The Minilab uses TLC procedures similar to those developed by Kenyon, Layloff, et al. except for crushing tablets inside aluminum foil instead of a plastic bag, development of layers and detection of spots in bottles instead of plastic bags, and detection with iodine vapor sublimed from crystals rather than KI-iodine solution. All necessary apparatus, reagents, and standards are included to test for 40 drugs on the WHO Essential Drug List (Table I). This list features antibiotics and chemotherapeutical agents frequently used in the developing countries of the southern hemisphere, which when counterfeited can be a serious threat to patients' lives [17]. The original TLC manual, dated 1998, and subsequent supplements (1999-2004) with detailed instructions covering analysis of the 40 drugs are available on the GPHF website [18].

Table I

Drugs analyzed by the Minilab TLC kit

Acetylsalicylic acid	Aminophylline	Amodiaquine	Amoxicillin
Ampicillin	Artemether	Artesunate	Cefalexin
Chloramphenicol	Chloroquine	Ciprofloxacin	Cloxacillin
Cotrimoxazole	Didanosine	Erythromycin	Ethambutol
Furosemide	Glibenclamide	Griseofulvin	Indinavir
Isoniazid	Lamivudine	Lumefantrine	Mebendazole
Mefloquine	Metamizole	Metronidazole	Nevirapine
Paracetamol	Phenoxymethylpenicillin	Prednisolone	Primaquine
Pyrazinamide	Quinine	Rifampicin	Salbutamol
Stavudine	Sulfadoxine/pyrimethamine	Tetracycline	Zidovudine

The first step in the Minilab protocol for screening counterfeit drugs is visual inspection of the product (e.g., size, shape, color) and its labeling and packaging, and comparison with a genuine example. 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 [4].

A dissolution and disintegration test is then carried out by dropping a tablet or capsule in warm (37°C) water contained in a 100 mL wide neck bottle and swirling periodically. Unless the product is labeled "slow release" or "enteric", it should disintegrate within 30 min, measured with a preset timer, or be suspected of being illegal.

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. An example is a colorimetric field assay for artesunate based on the reaction of fast red TR salt with an alkali decomposition product of the drug to produce a distinct yellow color [19]. Screening tests based on color reactions can be fooled by addition of another ingredient reacting the same as the active ingredient, or a small amount of the genuine pharmaceutically active substance, into the counterfeit drug product. In this case, a yes/no response is not adequate, and the method must be at least semiquantitative, like TLC.

In support of its TLC drug assays, the Minilab supplies a collection of authentic secondary standard tablets and capsules in sealed plastic tubes. The standard and the sample from a sachet or in the form of a hard gelatin capsule, soft gelatin capsule, or tablet are placed into glass bottles, and a designated volume of extraction solvent is added from a calibrated measuring pipet to prepare the stock solutions. The working solutions are prepared in 10 mL vials by appropriate dilution using pipets. The origin and mobile phase line are marked using a soft pencil on an aluminum backed 5 cm · 10 cm silica gel 60 F_{254} layer (called a "chromatoplate"), and disposable 2 μL glass micropipets are used to spot the standards and sample 1.5 cm up from the bottom edge. The uniformity of the initial spots (they should be circular and evenly spaced) is checked under a 254 nm UV lamp. Layers are developed in glass jars with lids and lined with filter paper on all sides; the mobile phase is added, and after 15 min of equilibration the jar is opened and the spotted layer is quickly inserted so that the initial spots are above the mobile phase level. After development up to the marked line (about three quarters of the layer length), the chromatoplate is removed, and the mobile phase is evaporated with the help of a hotplate (a travel iron placed upside down). The spots are viewed under battery-operated 254 nm and 366 nm UV lamps (Fig. 2). If necessary, spots not detected under UV light are detected as yellowish brown spots by placing the layer inside a capped jar containing iodine crystals (Fig. 3) and heating for about 30 s on the hot-plate. The

iodine detected spots and the spots seen under the UV lamps are marked with the soft pencil for documentation. All components of the kit must be thoroughly cleaned and solutions disposed of properly after each analysis.

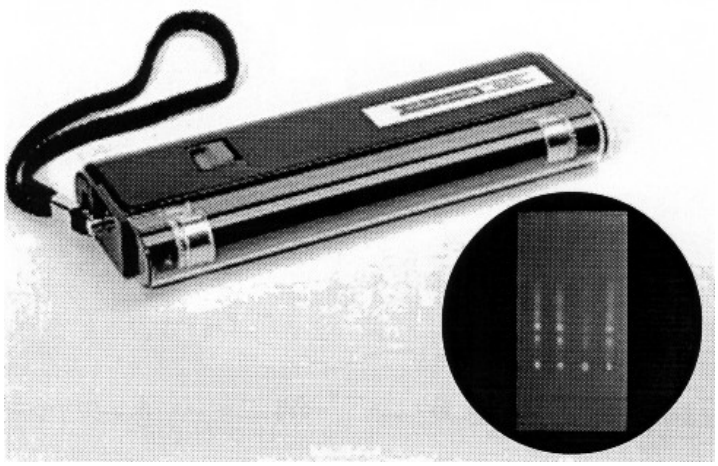

Fig. 2

Battery driven 366 nm UV lamp and chromatoplate with detected fluorescent spots (photograph supplied by GPHF)

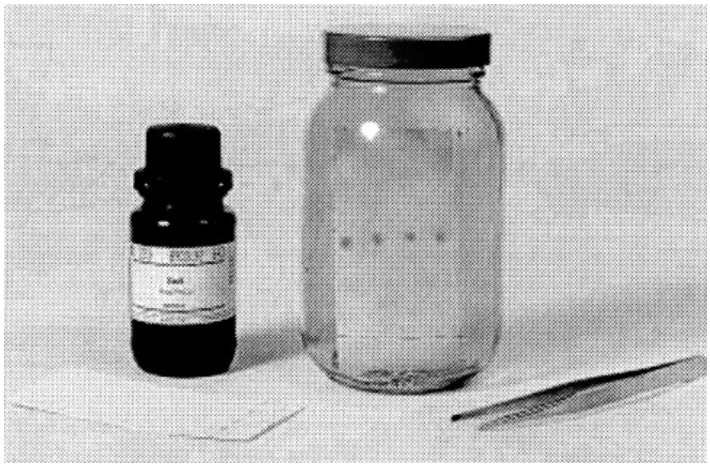

Fig. 3

Bottle of iodine crystals (left) and jar used as a tank for detection of spots by iodine vapor (right) with a chromatoplate inside. A second identical jar is used for development of the chromatoplate with mobile phase (photograph supplied by GPHF)

The Minilab TLC analysis identifies the active ingredient by comparison of distance of travel (R_F value) between the sample spot and an authentic standard spotted on the same plate, and semiquantitative proof of content 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 mobile 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.

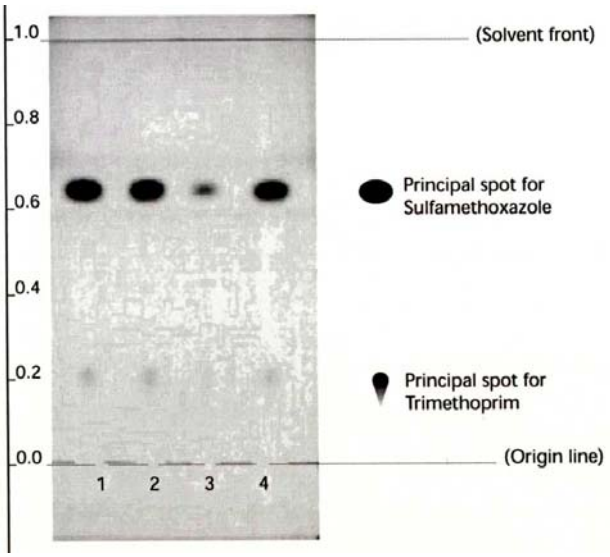

Fig. 4

Chromatoplate observed at 254 nm in the Minilab assay of cotrimoxazole; Lane 1, upper working limit representing 100% of total drug; Lane 2, a drug product of good quality; Lane 3, a drug product of poor quality; Lane 4, lower working limit representing 80% of total drug (photograph supplied by GPHF)

A proficiency test was carried out recently to assess the performance of Minilab visual TLC quantification estimates [20]. 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 identified. 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 inference on the quality of 45 samples. In both rounds, two inspectors failed to identify substandard samples. These results show the need to have competent, well trained users and to include a proficiency test in the Minilab screening program in order to obtain reliable results.

COMPLIMENTARY ANALYTICAL METHODS

The Minilab TLC method will not detect all of the other ingredients that are present in a sample, or identify or quantify any of the other ingredients that are detected unless corresponding standards are applied to the chromatoplate. For the most sophisticated counterfeit drugs, including those in which TLC detects some of the active ingredient but they still appear to be fakes, methods such as high performance column liquid chromatography (HPLC), gas chromatography (GC), nuclear magnetic resonance (NMR) spectrometry, mass spectrometry (MS), MS/MS, GC/MS, and HPLC/MS [21] are required for identification, and these methods can characterize and help find the manufacturers of counterfeit products.

A reversed phase (RP) HPLC method was developed, validated, and used to analyze potentially counterfeit antimalarial drugs (chloroquine, quinine, and mefloquine) purchased from the informal market in Congo, Burundi, and Angola [22]. RP-HPLC with acetonitrile-water (0.1% acetic acid) mobile phase and UV and electrospray ionization (ESI)-MS detection was applied to determination of seven pharmaceuticals (naproxen, ketoprofen, ibuprofen, diclofenac, piroxicam, nimesulide, and paracetamol) in counterfeit homeopathic preparations [23]. Sulfamethazine was identified as the active ingredient in counterfeit Halfan, an antimalarial drug, using accurate mass electrospray ionization MS, LockSpray MS/MS, and LC/MS [21]. Desorption electrospray ionization linear ion trap mass spectrometry (DESI-MS) is especially promising because it is a room temperature, open air surface characterization tool with no required sample preparation. Reactive DESI-MS [24] and DESI and direct-analysis-in-real-time (DART) coup-

led to time of flight (TOF)-MS [25] were recently demonstrated to be rapid screening assays for counterfeit artesunate antimalarials, and combined Fourier transform infrared (FTIR) imaging and DESI-MS was able to measure the distribution of all components on the surface of antimalarial tablets to aid in their detection and analysis [26].

Samples from 124 batches of counterfeit fenethylline (Captagon), a popular drug of abuse among the young in affluent communities of the Middle East, were analyzed by GC/MS. It was found that amphetamine, caffeine, and several other substances were present, but fenethylline was absent. The GC/MS method was able to make comparisons among the various samples and obtain inferences with respect to commonality of origin [27].

IR, Raman, and tetrahertz spectrometry can determine the overall composition of a drug product. The identities of active ingredients, excipients, and impurities and their relative proportions can provide spectrometric fingerprints that are helpful in authenticating a drug product [28]. The use of Raman spectrometry in the detection of counterfeit and adulterated pharmaceutical products was reviewed [29]. In a study of 50 supposed antimalarial artesunate tablets purchased in southeast Asia, Raman spectrometry was able to distinguish between genuine and counterfeit artesunate and confirm that the counterfeits contained no artesunate but mostly starch, calcite ($CaCO_3$), and paracetamol, or, in one case, a mixture of rutile (TiO_2) and artesunate [30]. It was demonstrated that noninvasive authentication of pharmaceutical products through packaging could be carried out with more sensitivity by spatially offset Raman spectrometry (SORS) compared to conventional backscattering Raman spectrometry; SORS was especially beneficial if the packaging, capsule shell, or tablet coating gave excessive surface Raman or fluorescence signals compared to weaker subsurface Raman signals of the active ingredients and excipients [31].

X-ray fluorescence spectrometry [32], inductively coupled plasma (ICP) atomic emission spectrometry [33], and ICP/MS [33] have been described for identifying the sources of illegal or unapproved drugs through the profile of trace metals they contain. Stable isotopic characterization by elemental analyzer-isotope ratio mass spectrometry (EA-IRMA) was shown to generate a very high degree of specificity for pharmaceutical materials in studies of their authenticity [34].

Near infrared (NIR) spectrometry is being increasingly used as a nondestructive method to detect pharmaceutical counterfeits with no sample preparation (see [35] for a review and [36] for a feasibility study). With appropriate choice of operating conditions, multiple samples can be com-

pared simultaneously, or detailed compositional information can be obtained and compared from individual samples [37]. As an example of the latest trend in instrumentation design, Fig. 5 shows the TruScan handheld NIR spectrometer (Ahura Scientific Inc., Wilmington, MA USA) that weighs less

Fig. 5

TruScan NIR handheld spectrometer being used to analyze a pharmaceutical through a plastic bag (Photograph supplied by Ahura Scientific, Inc.)

than 4 lb. "Point-and-shoot" operation provides pass/fail results for drug samples typically within 30 sec by comparison of the sample spectrum to a reference spectrum of the authentic product, with sampling through different types of containers such as tablets in blister packaging. An optional Discovery mode enables the operator to access a database library of drug and other chemical substances to determine the identity of an unknown substance that fails the initial test. Unlike TLC, NIR results are not quantitative, and the method is much more expensive for use by developing countries.

High performance electrophoresis was used for identification of Cordyceps (Chinese fungal pharmaceutical) and several kinds of counterfeits. A 70 μm x 60 cm quartz capillary column, 30 mmol L^{-1} borate buffer (pH 8.5), 20 kV voltage, and detection at 200 nm were used [38].

Polarized light microscopy can be used to examine solid dosage forms, identify excipients in a drug product, confirm a batch formula, and compare and differentiate between innovator, generic, and counterfeit products [39].

EPILOG

The TLC assays described above are a valuable aid in protecting patients from taking counterfeit and substandard quality medicines. They are more informative than visual inspection, dissociation tests, or simple color reaction tests, and their standardized format, ease of performance in the field by persons without extensive technical training, and low cost are of great benefit to developing countries throughout the world in screening medicines used for fighting diseases such as TB and malaria. More costly analyses in a fully equipped analytical laboratory are required only if the TLC screening results are ambiguous. Between the start of the program in 1997 and 2004, it was reported [5] that over 140 Minilabs were set up in more than 35 countries, and those numbers have certainly increased signify-cantly since 2004; this application of TLC is arguably the most significantly important one being carried out in the world today.

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