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Use of refractometry and colorimetry as field methods to rapidly assess antimalarial drug quality

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Abstract

The proliferation of counterfeit and poor-quality drugs is a major public health problem; especially in developing countries lacking adequate resources to effectively monitor their prevalence. Simple and affordable field methods provide a practical means of rapidly monitoring drug quality in circumstances where more advanced techniques are not available. Therefore, we have evaluated refractometry, colorimetry and a technique combining both processes as simple and accurate field assays to rapidly test the quality of the commonly available antimalarial drugs; artesunate, chloroquine, quinine, and sulfadoxine. Method bias, sensitivity, specificity and accuracy relative to high-performance liquid chromatographic (HPLC) analysis of drugs collected in the Lao PDR were assessed for each technique. The HPLC method for each drug was evaluated in terms of assay variability and accuracy. The accuracy of the combined method ranged from 0.96 to 1.00 for artesunate tablets, chloroquine injectables, quinine capsules, and sulfadoxine tablets while the accuracy was 0.78 for enterically coated chloroquine tablets. These techniques provide a generally accurate, yet simple and affordable means to assess drug quality in resource-poor settings.
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Keywords: Counterfeit drug; Drug quality; Antimalarial; Colorimetric; Refractive index

1. Introduction

Pharmaceutical counterfeiting and production of substandard drugs continue to burden the quality of health care worldwide. The World Health Organization has reported up to 25% of medicines consumed in developing countries are counterfeit or substandard [1]. These countries are most susceptible to this serious public health problem because of insufficient resources and infrastructure necessary to monitor and preserve drug quality. Since approximately 40% of the world's population is at risk of malaria [2], antimalarial drugs have become a particular favorite of counterfeiters [3–7]. The ability to identify counterfeit or poor quality pharmaceuticals is a critical component of a drug quality assurance system. Product quality is typically evaluated using the specifications and methods described in pharmacopeias, but the analytical techniques used, generally involve sophisticated instrumentation such as high-performance liquid chromatography (HPLC), spectrophotometers and dissolution apparatus. These instruments usually require periodic maintenance, highly trained personnel and a controlled laboratory environment for proper functioning. Unfortunately, these resources are often lacking in countries where counterfeit drugs are common. Since counterfeit drugs have been increasingly recognized as a significant public health issue, more inspection and random testing are required in these financially poor countries. Until, more effective testing procedures are implemented, sim-

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ple and affordable field methods provide a practical means of rapidly monitoring drug quality. Field methods should be robust, inexpensive, portable, simple to conduct and reasonably accurate. Minimal use of toxic or flammable reagents is desirable.

Drug quality assessments initially include organoleptic inspection of the product followed by a determination of the amount of proper active pharmaceutical ingredients (API) present in the sample by commonly used basic tests such as colorimetry and thin-layer chromatography (TLC). Colorimetric techniques aid in the identification of particular active ingredients by making use of color changes produced by specific chemical reactions. The color changes are usually rapid and easily discernable. Quantitative measurements of active ingredient concentration as a function of color absorbance can be made using a portable battery-powered photometer. Although less specific than TLC, colorimetry does not require a separation phase requiring flammable or toxic organic solvents. A number of colorimetric tests for antimalarials and other essential drugs have been published [8–12].

Common physical properties of matter (bulk properties) such as weight, density, refractive index, viscosity, crystal morphology and solubility can be used to identify counterfeits [13]. The equipment required for measuring these properties (e.g. balance, refractometer, hydrometer, magnifying glasses or microscopes) are relatively inexpensive, portable, and rugged. For example, it has been shown that a simple refractometer can be used to monitor tampering of controlled substances by measuring the refractive index of a drug solution [14,15].

The objective of this study was to evaluate the use of two techniques, refractometry and colorimetry as separate and complimentary field methods to rapidly assess the quality of chloroquine injectables, chloroquine (EC) enteric-coated tablets, quinine capsules, sulfadoxine/pyrimethamine (SP) tablets and artesunate tablets. The previously reported colorimetric tests have been adapted for use as an adjunct to the refractive index methods. The adaptations include a modification of the extraction component for each technique. Extracts of the active ingredients from each solid dosage form or injectable solutions were subjected to refractive index (RI) measurements and a specific colorimetric assay. The results were compared with high-performance liquid chromatographic (HPLC) analysis.

2. Materials and methods[1]

2.1. Reagents and apparatus

All reagents used were of analytical-reagent grade and deionized water used for all aqueous solutions. Reference standards were 97–103% purity and prepared at 25 mg ml^{-1}. All dye solutions were freshly prepared.

Artesunate reference standard (gift from Mepha, Aesch-Basel, Switzerland) was prepared in isopropanol–methanol (1:9, v/v). Reagents for the colorimetric test include 1.1 M acetic acid, 1N sodium hydroxide, and an aqueous solution of 5 mg ml^{-1} fast red TR salt, dye content ~20% (Sigma, St. Louis, MO, USA).

Chloroquine phosphate reference standard (Sigma) was prepared in water. Reagents for the colorimetric test include 0.1 M potassium dihydrogen phosphate, 0.1N sodium hydroxide, ethyl acetate, and a 1 mg ml^{-1} aqueous solution of bromochlorophenol blue salt (Sigma).

Quinine sulfate reference standard (Sigma) was prepared in 1.7% phosphoric acid. Reagents for the colorimetric test include 1.1 M acetic acid, 1N sodium hydroxide, ethyl acetate, and an aqueous solution of 1 mg ml^{-1} Congo Red (Sigma).

Sulfadoxine reference standard (gift from Hoffman-LaRoche, Basel, Switzerland) was prepared in ammonium hydroxide–isopropanol–methanol (5:9.5:85.5, v/v/v). A solution of *p*-dimethylaminocinnamaldehyde (Sigma) at 0.25 mg ml^{-1} in 1.1 M acetic acid is used as the colorimetric reagent.

Other compounds used to evaluate assay selectivity include tetracycline hydrochloride, chloramphenicol, pyrimethamine (Sigma–Aldrich, St. Louis, MO, USA), amoxicillin, ampicillin, erythromycin, acetylsalicylic acid, acetaminophen, ciprofloxacin hydrochloride (ICN Biomedicals, Inc., Aurora, OH, USA), amodiaquine hydrochloride (Park-Davis Co., Detroit, MI, USA) and mefloquine hydrochloride (Hoffman-LaRoche, Basel, Switzerland).

Refractive index measurements were obtained using a hand-held battery-powered AR200 digital refractometer (Leica Microsystems, Buffalo, NY, USA). Absorbance measurements were taken using a Spectronic 21 spectrophotometer (Milton Roy, Reviera Beach, FL, USA). HPLC analysis was conducted with an Agilent 1100 Series system (Agilent, Palo Alto, CA, USA) using a 150 mm × 4.6 mm, C18 column and a mobile phase consisting of acetonitrile and 0.05 M perchlorate (pH 2.5) flowing through the column at a rate of 1 ml min^{-1}. Column temperature was maintained at 30 °C. Drug components were detected by UV absorbance at 254 and 280 nm.

2.2. Procedures

2.2.1. Sample processing

Samples consisted of artesunate tablets, chloroquine phosphate (EC) tablets, chloroquine phosphate injectables, quinine sulfate capsules, and sulfadoxine/pyrimethamine (SP) tablets. The samples were collected in Lao PDR (Laos) and represent commonly available antimalarial drugs. Each tablet was enclosed in a sleeve of aluminum foil, thoroughly pulverized with a pestle, and the contents transferred to a glass vial. Chloroquine EC tablets were soaked in 0.1 M sodium hydroxide for 5–10 min to remove the enteric coating prior to pulverization. One of four extraction solvents was used to solubilize the API from each preparation. These solvent mixtures include isopropanol–methanol (1:9, v/v) for artesunate, ammonium hydroxide–isopropanol–methanol (5:9.5:85.5, v/v/v) for sulfadoxine, 1.7% phosphoric acid for quinine sulfate, and water for chloroquine phosphate and were chosen for their ability to solubilize each API to a concentration of at least 25 mg ml^{-1}.

[1] The use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the US Department of Health and Human Services.

The amount of API for each drug (as declared by the manufacturer) was noted and a sufficient volume of solvent was added to the entire sample to achieve an alleged final API concentration of 25 mg ml^{-1}. All artesunate tablets were labeled as containing 50 mg of API, chloroquine phosphate tablets and quinine sulfate capsules, 250 mg of API and SP tablets, 500 mg of sulfadoxine and 25 mg of pyrimethamine. Chloroquine injectables (322.5 per 5 ml of sterile water) were analyzed directly and therefore not processed. Drug mixtures were shaken vigorously for 10 s, allowed to equilibrate for 10 min, shaken again, and filtered through 0.22 or 0.45 μm nylon or PFTE membrane.

2.2.2. HPLC analysis

Chloroquine, quinine, and sulfadoxine sample extracts were diluted 1/100 and artesunate diluted 1/10 with 50% acetonitrile before the injection of 5 μl into the HPLC system. Mobile phase consisting of 60% acetonitrile was used for artesunate analysis while 30% acetonitrile was used for chloroquine, quinine, and sulfadoxine analysis. Absorbance detection at 254 and 280 nm were chosen since they are common filters for inexpensive single-wavelength UV detectors. The retention times for each drug ranged from 3 to 6 min. API concentrations were determined by comparison with the appropriate reference standard using absorbance measurements at each wavelength, 254 and 280 nm. The final concentration was determined from the average of concentrations calculated from each absorbance wavelength. Any large discrepancy in calculated concentrations for each wavelength would indicate an interfering component or wrong API. Absorbance ratios (254/280 nm) for each reference standard were determined for artesunate (2.9), chloroquine (9.5), quinine (15.7), and sulfadoxine (0.9). The absorbance ratios for each sample were monitored as an indicator of component peak purity. The HPLC methods used a common reverse-phase separation column, mobile phase, and detection wavelengths to facilitate a simple procedure for use with a variety of essential drugs. Validation of the HPLC methods in terms of linearity, assay precision and accuracy was performed using pharmaceutical preparations containing known amounts of active ingredient. These preparations were composed of a generic excipient mix (69% microcrystalline cellulose, 20% croscarmellose sodium, 5% talc, 5% magnesium stearate, and 1% fumed silicon dioxide), the API and lactose. For example, an average chloroquine tablet in our sample group weighed ∼500 mg and contained 250 mg of API. A chloroquine tablet preparation therefore consisted of 250 mg of chloroquine phosphate, 100 mg excipient mix and 150 mg lactose as filler. The excipient mix was kept constant while various ratios of API and filler was added to produce preparations containing 0, 20, 50, 80, 100, 120, and 150% of the amount of API normally found in the tablet. Enough solvent was added to the "100% API" sample to achieve a concentration of 25 mg ml^{-1}. The same volume was added to subsequent samples achieving the following concentrations (mg ml^{-1}): 5.0 (20%), 12.5 (50%), 20 (80%), 25 (100%), 30 (120%), and 37.5 (150%). These concentrations were evaluated for linearity, precision (% standard deviation) and accuracy (100 × differences between nominal concentration and observed concentration divided by the nominal concentration) for five different preparations.

2.2.3. Colorimetric and refractometric analysis

Sample extracts were applied directly to a refractometer and measurements made using a non-temperature-compensated Brix scale. Since refractive index is temperature-dependent, samples and reference standards were measured at approximately the same temperature, ±2 °C. A corresponding solvent blank was also measured and the value subtracted from the sample values. The difference in the extract and corresponding solvent (blank) was compared to an equivalent concentration of the associated reference standard to determine sample concentration. A portion of the extract was also subjected to HPLC analysis to evaluate the validity of the RI method in terms of method bias [16], assay sensitivity, specificity and accuracy.

All colorimetric reactions and absorbance measurements were conducted using siliconized 13 mm diameter glass tubes. Linearity of the colorimetric assays for each drug was determined by a linear regression plot of the volume (μl) of reference standard solution (25 mg ml^{-1}) and the average absorbance measurements of five samples. A measured volume from each sample extract was subjected to quantitative colorimetric tests as described below and compared with a known concentration of reference standard solution. Corresponding "blanks" (excipients) were prepared with no API and the absorbance measurements subtracted from the samples and reference standard measurements. Drug concentrations were determined by comparison with reference standards prepared at a concentration of 25 mg ml^{-1}. Method bias, assay sensitivity, specificity, and accuracy were evaluated by comparison of the colorimetric results with HPLC analysis. API amounts were determined quantitatively for each sample and regarded as a "positive" for a counterfeit or poor quality drug if the %API (API determined by assay divided by API declared by the manufacturer × 100) is outside the range of 80–120%.

The colorimetric test for artesunate is a modification of that described by Green et al. [11]. Twenty microliters of reference standard or tablet extract was added to 0.5 ml of 1N sodium hydroxide, mixed and allowed to react for 5 min (rapid test). A reaction time of up to 20 min may be used for increased sensitivity. One milliliter of 1.1 M acetic acid and 0.5 ml of FRTR salt was added to the mixture allowing a reaction time of 5 min. A yellow color is observed when artesunate or dihydroartemisinin is present. Absorbance measurements were taken at 420 nm. To minimize false positives with artemisinin or sulfadoxine, it is important that the pH of the reaction buffer is 4–5 [11].

The colorimetric test for chloroquine is a modification of that described by el-Ashry et al. [9]. A pH 8 buffer was prepared by mixing 0.1 M potassium dihydrogen phosphate and 1N sodium hydroxide (50:46.7, v/v). Thirty microliters of the standard or sample extract was added with 1.0 ml of bromochlorophenol blue solution. Three milliliter of ethyl acetate was added and the samples were vigorously shaken. After phase separation, the top layer was transferred to a clean siliconized glass tube and the absorbance measured at 580 nm. The top, organic layer is blue if chloroquine is present.

A colorimetric method specific for quinine and mefloquine was described Green and Wirtz [17]. Ten microliters of quinine sample extract or standard and 140 microliters of Congo Red

solution was added to 2 ml of pH 5 buffer (1.1 M acetic acid: 1N sodium hydroxide (1:1, v/v). Three milliliter of ethyl acetate was added and the sample vigorously shaken. After phase separation, the top organic layer (red if quinine or mefloquine is present) was transferred to a clean glass tube for absorbance measurements at 490 nm.

The procedure used for the colorimetric sulfadoxine assay is a modification of that described previously [18,19]. Fifty microliters of SP tablet extract was added to 3 ml of *p*-dimethylaminocinnamaldehyde solution. A burgundy color immediately develops in the presence of sulfadoxine or any other sulfa drug possessing an aromatic amine group [20].

3. Results and discussion

3.1. HPLC analysis

The HPLC analyses were used as reference methods in assessing the colorimetric and refractometric techniques. Therefore, evaluation of the HPLC methods in terms of accuracy, precision, and linearity are shown in Table 1. The accuracy of the methods ranged from 92 to 106% for all drugs at all concentrations. Precision was satisfactory (<15%) for chloroquine, quinine, and S/P.

The variability for the artesunate preparations was higher (≤20%) than the other antimalarials, possibly resulting from measurement error due to the smaller proportion of API (50 mg/sample) relative to the excipients as compared with the other drugs (250–500 mg/sample). The concentration curves were linear for all drugs (average $r^2 > 0.988$ for absorbance detection at 254 and 280 nm).

3.2. Colorimetric and refractometric analysis

Regression analysis plots for each colorimetric assay (Fig. 1), revealed linear relationships in accordance to Beer's law for each specified volume range. Quantitative colorimetric assay biases (HPLC/test) for all antimalarials tested were not significantly different from unity, although chloroquine EC tablets exhibited higher variability (Table 2). Chloroquine EC tablets also showed a significant bias of 0.72 for the RI methods. Since water is used to dissolve the active ingredients (API) and the tablets are treated with sodium hydroxide prior to pulverization, it is likely dissolved excipients as well as residual sodium hydroxide may increase the RI measurements. Therefore, in subsequent evaluations of assay sensitivity and specificity for chloroquine EC tablets, concentrations derived from RI measurement were multiplied by 0.72 to compensate for interference.

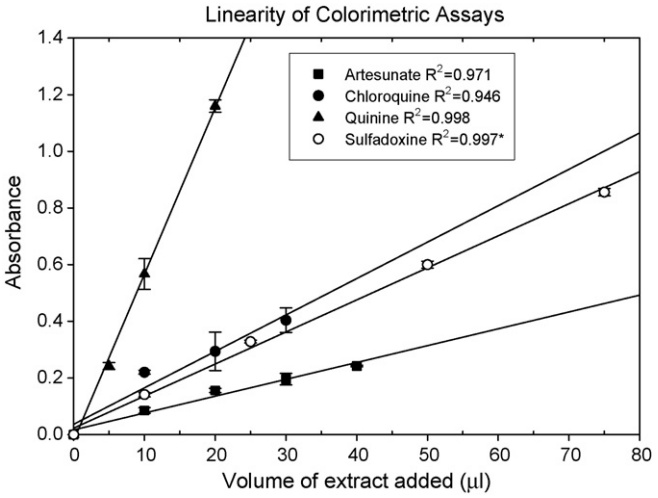

Fig. 1. Linear regression analysis for the colorimetric assay of each antimalarial drug. Average absorbance values and standard deviation bars were determined from five samples. Asterisk (*) indicates sulfadoxine samples were diluted 1:1 with water before absorbance measurements were made.

Table 2
Ratio of HPLC assay results against quantitative colorimetric and refractometric determination of API concentration (bias = HPLC/field test)

Drug	Average bias ± S.D. (*n*)	
	Refractometry	Colorimetry
Artesunate tablets	0.91 ± 0.18 (52)	0.95 ± 0.04 (27)
Chloroquine tablets	0.72 ± 0.10 (108)	1.24 ± 0.35 (108)
Chloroquine injectables	1.03 ± 0.06 (58)	1.11 ± 0.06 (58)
Quinine capsules	0.93 ± 0.14 (147)	1.19 ± 0.16 (69)

Table 3 shows assay sensitivity, specificity, and accuracy for the colorimetric and RI tests. The accuracy of a method is defined as the proportion of tests that give correct results [(true positives + true negatives)/all tests]. API measurements outside the range of 80–120% were regarded as positive for a poor quality drug. Medicines from the WHO essential drug list were added as controls positive for a counterfeit or "wrong drug" in order to determine selectivity. These include amodiaquine, mefloquine, erythromycin, tetracycline, acetaminophen (paracetamol), acetylsalicylic acid (aspirin), chloramphenicol, amoxicillin, ampicillin, ciprofloxacin, and pyrimethamine.

Table 1
Accuracy and precision of HPLC methods for the analysis of pharmaceutical preparations of artesunate, chloroquine, quinine and sulfadoxine (*n* = 5)

Nominal concentration (mg ml^{-1})	Artesunate		Chloroquine		Quinine		Sulfadoxine	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
5.0	103	17	92	2	93	2	97	10
12.5	98	12	97	2	99	3	95	7
20.0	97	18	97	1	100	7	102	13
25.0	99	20	97	5	106	4	110	6
30.0	98	10	100	1	98	3	95	6
37.5	103	7	103	2	99	1	99	4

Table 3
Validation of RI, colorimetric and "RI + colorimetric" assays relative to HPLC reference standard

Drug	*n*	Method	Sensitivity	Specificity	Accuracy
Artesunate (50 mg per tablet)	111	RI	0.86	0.87	0.86
		Color	0.79	1.00	0.95
		RI + color	0.86	1.00	0.96
Chloroquine phosphate (250 mg per EC tablet)	119	RI	0.83	0.73	0.76
		Color	0.50	1.00	0.81
		RI + color	0.83	0.75	0.78
Chloroquine phosphate (322.5 mg per injectable)	73	RI	1.00	0.86	0.97
		Color	0.97	1.00	0.97
		RI + color	1.00	1.00	1.00
Quinine sulfate (250 mg per capsule)	80	RI	0.98	0.56	0.96
		Color	0.94	0.94	0.96
		RI + color	0.98	0.88	0.96
Sulfadoxine (500 mg per tablet)	75	RI	0.97	0.64	0.91
		Color	0.79	1.00	0.83
		RI + color	0.97	1.00	0.97

RI and color tests were quantitative. If API (amount measured/amount declared by manufacturer × 100) is outside the range of 80–120%, the sample is regarded as "positive" for a counterfeit or poor quality drug. The RI values for chloroquine EC (enteric coated) tablets were adjusted by a factor of 0.72 (bias associated with the RI method). The color tests in the "color + RI" method were qualitative, i.e. absorbance values >0.1 were considered to be confirmatory for the presence of the suspected active ingredient. Sulfadoxine tablets also contain 25 mg of pyrimethamine per tablet.

Assay sensitivities (0.83–1.00) revealed few false negatives for all drugs using the RI testing methods although assay specificities were poor for quinine sulfate (0.56) and sulfadoxine (0.64). Assay specificity (0.86) was better for artesunate since it is completely soluble in alcohol at 25 mg ml^{-1} relative to chloroquine phosphate, quinine sulfate and sulfadoxine, which are insoluble or slightly soluble.

For the quinine RI test, false negatives were observed with amodiaquine, ciprofloxacin, tetracycline, ampicillin, and chloroquine while aspirin, acetaminophen, ampicillin, amoxicillin, and quinine showed false positives for the sulfadoxine RI test. Specificity was improved for APIs soluble in water (chloroquine test, where false positives include ciprofloxacin and tetracycline) and alcohol (artesunate test, where false positives include mefloquine, aspirin, and quinine).

Quantitative determination of API using the colorimetric methods showed good specificities (few false positives), although the sensitivities were lower than those for the RI method. The colorimetric techniques were very selective in identifying the proper API. Although the yellow color of amodiaquine interfered with the artesunate colorimetric assay (yellow if positive for artesunate), the amount of amodiaquine per tablet (250 mg) indicated a counterfeit or substandard drug if 50 mg/tablet was expected for an artesunate tablet. Sensitivity (0.97) and specificity (1.00) were very good for the chloroquine injectables, but the sensitivity (0.50) for the chloroquine EC tablets was poor. Although the quinine colorimetric test gave a positive color reaction with mefloquine, this drug has much lower solubility in the 1.7% phosphoric acid extraction solvent than quinine sulfate, therefore exhibiting a much lower absorbance and low apparent concentration.

The combination of RI and colorimetry in terms of sensitivity, specificity, and accuracy was also evaluated. The RI method reflected the quantitative aspect while the colorimetric test reflected a qualitative aspect of the combined (RI + color) test. Absorbance values generally above 0.1 reveal a distinct observable color; therefore any value below this threshold was regarded as a poor quality drug. If the quantitative RI test showed %API levels to be within the specified range of 80–120% and the absorbance of the colorimetric test were above 0.1, then the sample was considered to be of good quality. Alternatively, if the colorimetric absorbance is <0.1 or the %API, as determined by RI, is outside the 80–120% range, the sample is considered to be of poor quality. Therefore, a combination of the two methods resulted in acceptable accuracy (Table 3). Although, the RI method is not selective for a particular API, the qualitative colorimetric technique provides the selectivity associated with the confirmation of the appropriate drug while the RI method confirms the proper amount of API.

4. Conclusions

Highly accurate and specific analysis methods require sophisticated and expensive instrumentation as well as experienced personnel for operation and maintenance. In many circumstances, these resources are not available, resulting in inadequate oversight of drug quality. As a consequence, counterfeit and poor quality drugs will continue to proliferate. Although, the accuracy and selectivity of simple and inexpensive techniques are less than more sophisticated techniques, these methods have the advantage of convenience and affordability as well as the ability to perform analysis on a large number of samples in remote environments. Conditions such as temperature, humidity, and reagent quality can be quite variable in these environments. Therefore it is advisable that these tests be conducted in tandem with positive (authentic reference standard) and negative controls to confirm the integrity of the assay.

The main objective of this study was to evaluate the practicality of using colorimetric and RI methods as a rapid, simple, and affordable method to monitor and screen for poor quality drugs. A combination of the RI method (quantitative) and a qualitative colorimetric method was shown to provide an accurate technique for determining drug quality. The technique is field-friendly and only requires a refractometer for quantitative measurements. Since RI is a measure of all soluble components, the accuracy of this method is dependent on API and excipient solubility in the specified solvent. For example, the RI analysis of chloroquine EC tablets tended to be 1.4 times higher, while the bias for chloroquine injectables, artesunate tablets, quinine capsules, and S/P tablets were not significantly different than unity. Since the RI method cannot distinguish between various drugs, the specific colorimetric assays were incorporated as a qualitative test to confirm the presence or absence of the drug being analyzed. Although, portable photometers can quantitatively measure color absorbance as a function of drug concentration, the use of hand-held prism-type refractometers to measure API concentrations provide a rugged, affordable, and portable means of analysis. Small amounts of active ingredient in counterfeits will give a positive color reaction, therefore a quantitative aspect of the analysis using either refractometer (density) or a photometer (color absorbance) should be used to determine if the amount of API is consistent with that declared by the manufacturer. A large variety of field techniques, such as organoleptic inspections of product, TLC, colorimetry, RI, solubility, crystal morphology and pH can easily be adapted or combined according to the situation the drugs are being tested under, to provide developing countries with an assortment of techniques to aid in identifying poor quality pharmaceuticals. These methods can provide a preliminary assessment of drug quality until circumstances provide for more sophisticated analysis methods.

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