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Lab on Paper: Assay of Beta-Lactam Pharmaceuticals by Redox Titration

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Lab on Paper: Assay of Beta-Lactam Pharmaceuticals by Redox Titration

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ABSTRACT

Field tests to detect substandard antibiotics are sorely needed in many low and middle-income countries (LMICs). We created a field screening test for amoxicillin capsules and ampicillin tablets based on USP method <425>. To measure the amount of antibiotic, the user dissolves the contents of the capsule in water, carries out a simple chemical degradation, and adds drops of the sample to a paper test card. The test card performs a titration and displays a series of blue dots for visual comparison to standard images.

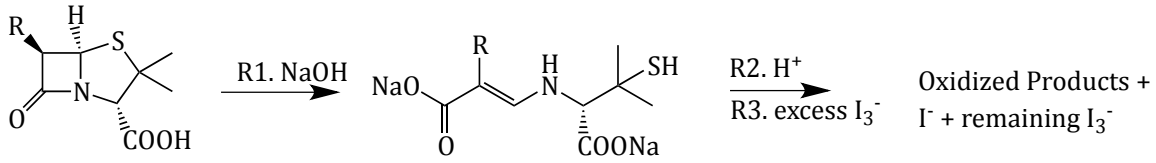
Pharmaceutical samples were collected in western Kenya and analyzed using a blinded methodology to validate the test card's quantitative performance against HPLC assay. Within the quantification range of the test card, the absolute assay error for dosage forms was 4.4% for amoxicillin and 5.3% for ampicillin, and inter-operator precision was 2%. Users correctly categorized 94% of amoxicillin (n=84) and 88% of ampicillin (n=56) samples as either containing less than or greater than 90% of the expected amount of antibiotic, which is the lower regulatory limit. The test card detected several lot numbers of a substandard amoxicillin product, confirmed by HPLC analysis to contain only 40-60% of the stated amoxicillin content. IR, PXRD, and gravimetric analysis revealed that talc made up the remainder of the adulterated medications.

BACKGROUND

The quality of beta-lactam antibiotics is a matter of life or death, so detecting bad quality products in low- and middle-income countries (LMICs) is an important analytical goal. Pneumonia kills more than 20,000 children under age 5 every year in Kenya.[1,2] National medical guidelines recommend that children with community-acquired pneumonia receive amoxicillin, an inexpensive beta-lactam antibiotic.[2] The World Health Organization (WHO) reports that 10% of the antibiotics in low and middle-income countries (LMICs) are substandard or falsified (SF), and SF amoxicillin is estimated to kill 72,000 children per year in sub-Saharan Africa.[3]

Medicines must meet quality criteria enforced by drug regulatory agencies (DRAs). These quality standards cover a range of properties that relate to the efficacy of the product, ranging from the weight and purity of the ingredients to the tablet color, uniformity, and dissolution rate. One critical standard is the active pharmaceutical ingredient (API) content, because if the medicine does not contain the correct amount of the antibiotic, the concentration in the patient's blood will not reach the therapeutically effective level, which can contribute to development of resistant pathogens. For amoxicillin and ampicillin, the API content of a dosage form must assay between 90% and 110 or 120% of the quantity stated on the package.[4,5] Boehle et al.[6] developed a paper-based enzyme competition assay that responds to multiple beta lactam-containing penicillin and cephalosporin antibiotics. This device has the potential to identify products that are severely underdosed (<70% API content), but it is yet not accurate enough to identify products that fail to meet pharmacopoeia standards.

The United States Pharmacopeia (USP) contains a method, <425> "Iodometric assay-antibiotics," that quantifies fourteen beta-lactam antibiotics by a back-titration (Figure 1).[7] The beta-lactam antibiotic is degraded in base for 15 minutes to generate redox active species. The complete degradation pathways are complex,[8] so only one product is shown in Figure 1 to simplify the explanation of the assay. The solution is acidified, and an accurately known excess of triiodide is added to oxidize the degradation products. The solution reacts for another 15 minutes, and whatever triiodide remains is back-titrated with thiosulfate using starch as an endpoint indicator.



R4. $I_3^- + Na_2S_2O_3 \leftrightarrow I^- + Na_2S_4O_6$

R5. I_3^- + starch ←→ blue complex

Figure 1. Back-titration of beta-lactam antibiotics. R1. The antibiotic is degraded with base to produce redox active species, reaction time = 15 min. R2. The solution is acidified. R3. A known excess of triiodide is added, reaction time = 15 min. R4. The remaining triiodide is quantified via iodometric titration. R5. The starch indicator will be colored blue if the oxidizing capacity of the triiodide exceeds the reducing capacity of the thiosulfate. If not, the indicator will be colorless.

We previously reported a test card that performs an iodometric titration for analysis of iodized salt, and demonstrated it could quantify pure amoxicillin through the back-titration method.[9] Here, we apply this method to dosage forms of amoxicillin and ampicillin, and assess the accuracy of the card through a blinded validation study using samples collected from the Kenyan marketplace in 2012-2014.

EXPERIMENTAL

Pharmaceutical sample collection in Kenya

Overview

Ethical clearance for the study was obtained from the Moi University/Moi Teaching and Referral Hospital Institutional Research and Ethics Committee (IREC # 2012/45) and from the University of Notre Dame (IRB #14-04-1763 and 15-05-2542). Ethical issues considered included the use of deception (covert shoppers), privacy of drug vendors, and arrangements for reporting suspicious samples to the Kenyan Pharmacy and Poisons Board, which is the drug regulatory agency in Kenya, and the WHO Rapid Alert system, which tracks global pharmaceutical quality problems.

The pharmacovigilance department at Moi Teaching and Referral Hospital in Eldoret, Kenya trained secret shoppers to collect medicines from the marketplaces of 21 nearby towns: Busia, Port Victoria, Ugunja, Kakamega, Malaba, Webuye, Sio, Nangili, Matunda, Moi's Bridge, Kitale, Kisumu, Bungoma, Luanda, Nakuru, Nairobi, Mombasa, Kapsabet, Mosoriot, Turbo and Kipkaren River. These sites were selected based on the recommendation of the Kenyan Pharmacy and Poisons Board as sites with a higher potential for substandard medications. Details of how the secret shoppers were trained and how pharmacies and medicines were selected are in the Electronic Supporting Information (S6).

Sample storage

Samples were assigned unique codes, and each sample's API, dosage, manufacturer, distributor, expiry date, batch number, and type of packaging along with the presence/absence of an insert was recorded in an Excel spreadsheet. They were stored at Moi Teaching and Referral Hospital (MTRH) at ambient temperature away from sunlight and water. The samples were shipped by air to the University of Notre Dame (UND) and then refrigerated at 4 °C until they were analyzed.

Materials. Chemicals used included soluble starch (J.T. Baker), p-toluenesulfonic acid (Alfa Aesar), potassium iodide (Amresco), cadmium chloride (Acros), anhydrous sodium thiosulfate (J.T. Baker), secondary standard of potassium iodate, 100.2% (J.T. Baker), primary standard of amoxicillin trihydrate (USP), secondary standard of amoxicillin trihydrate (Sigma Aldrich), primary standard of ampicillin (USP), secondary standard of ampicillin (Sigma Aldrich), standardized 0.005 M iodine solution (Alfa Aesar), hydrochloric acid (Fisher), and sodium hydroxide (Fisher). The PADs were fabricated using Ahlstrom 319 paper (8.5 x 11" sheets custom ordered via Midland Scientific, Chicago, IL), and were printed on a ColorQube 8570N wax printer (Xerox). Solutions of 1.0 M NaOH and 1.2 M HCl were stored in polyethylene bottles, and 0.0050 M triiodide was stored in glass vials with Teflon® caps. All amoxicillin concentrations are reported as anhydrous amoxicillin.

Fabrication of test card. The test card was created by printing solid wax ink onto Ahlstrom 319 paper using a Xerox ColorQube printer; details and the Adobe Illustrator print files are available.[9] The cards were baked at 100°C for 14 minutes. The seal was tested on a small number of the cards by placing water into a zone, waiting 30 seconds, and seeing if water leaked into an adjacent zone. If it did, then the batch of cards was

baked for 3 more minutes, retested, and repeated until the seal was good. The reagents listed in Table S3 were deposited with a multichannel automatic pipette into the locations specified in Figure S1.

Stock solution preparation for test card and HPLC analysis. Standard solutions were prepared using deionized water and primary or secondary reference materials traceable to USP standards. For the internal validation study, samples were weighed on an Explorer semi-micro balance, model OHAUS EX125D, which has a 12 mg minimum weight as defined by USP <41>. At least 25 mg of each sample was taken for assay. A nominal 2.0 mg/mL stock of amoxicillin or ampicillin was prepared in deionized water. An aliquot of the stock was used to create a 1.00 mg/mL solution for the test card analysis. A separate aliquot was taken from the stock to create a 0.5 mg/mL solution for HPLC analysis. This was done so capsule-to-capsule heterogeneity could not cause different measurements between the methods. For the standards used to generate the paradigmatic test card images used to analyze the other samples, the concentration of amoxicillin or ampicillin in the reference solutions varied from 0.80 to 1.10 mg/mL in 0.05 mg/mL increments.

Mocked-up amoxicillin samples. The contents of 20 capsules of one lot of an amoxicillin dosage form (Caremox-500 lot 160927, labeled as containing 500 mg anhydrous amoxicillin as amoxicillin trihydrate) were mixed in a 50 mL Falcon tube and vortexed for 4 minutes. The total mass of the powder was corrected for the weight of powder retained in the capsules (about 2% retained). The retained powder was determined by weighing the gel capsule shells before and after blowing all the powder out with compressed air. The actual content of amoxicillin in this lot number was assayed by HPLC as 85 ± 1%. Accurately massed portions of the dosage form and talc or starch

were placed into a Falcon tube or a glass vial and mixed for 4 minutes using a vortexer and inverting the container regularly. The chemist who diluted the samples blinded them and did not perform any of the test card or HPLC analysis.

HPLC system suitability. Identical HPLC systems are installed in the UND lab and in the MTRH lab and the two labs can communicate via videoconferencing software. Samples were analyzed using a Waters 2695 HPLC with a Waters 2487 absorbance detector set at 220 nm. The detector is monitored by an SRI model 333 A to D converter (SRI Instruments, Torrance, CA, USA), connected to a laptop computer running PeakSimple (SRI Instruments, Torrance, CA, USA). Peak integration data from PeakSimple is manually transcribed into an Excel template,[10] which performs the necessary standardization, calibration checks, and concentration calculations.

A complete system suitability assessment according to USP <621> was performed. The assay met USP standards for peak metrics, linearity, precision, accuracy, resolution, and recovery of the beta lactam antibiotic from a matrix of degraded capsule contents. Before each batch of unknowns was analyzed, five replicate injections of the reference standard were run. If these replicates produced less than a 2% relative standard deviation for reproducibility, the unknowns were run. A calibration check sample (prepared each day from a secondary standard of amoxicillin or ampicillin) was run after every 5 unknowns, and the 5 runs were rejected if the integrated intensity of the calibration check peak fell outside the 2% RSD range established at the start of the run. Due to the small number of capsules available in each sample for assay and the need to retain capsules for analysis by the national drug regulatory agency, single-capsule or single-tablet analysis was performed (most pharmacopeia procedures call for homogenizing the contents of 10 or

more capsules for assay). The standard operating procedure for any capsule that failed to meet USP standards (90%-120% API content) was to analyze the original capsule again and two additional capsules taken from the same package. Samples that failed these tests were reported to the Kenyan Pharmacy and Poisons board.

Preparation of samples for aPAD and HPLC analysis. For capsules, the powder was massed by difference using an airstream to blow out the capsules until no residual powder could be seen. Tablets were weighed and then ground to powder in a mortar. samples were weighed on an Explorer semi-micro balance, model OHAUS EX125D, which has a 12 mg minimum weight as defined by USP <41>. At least 25 mg of each sample was taken for assay and diluted to nominal 1.00 mg API/mL in deionized water for analysis on the test card or diluted to 0.5 mg API/mL in deionized water for HPLC analysis, which was conducted on the same day. The column used was a Symmetry C18 5µm, 4.6 x 100 mm column. The 18 µl of sample was injected via autoinjector. The details of the HPLC conditions used for each antibiotic are shown in supporting information, section **S3**.

Analysis of samples on aPAD: The rate of oxidation of the amoxicillin degradation products by triiodide was found to be temperature-dependent (supporting information, section S8). Temperatures of 15˚C or below give incomplete reaction after 15 min reaction time, leading to an underestimate of more than 5% in the antibiotic content in 100% API content samples, while temperatures of 30˚C or above yield an overestimate of more than 5% in the antibiotic content. The following steps were performed in a scintillation vial at lab room temperatures of 20-25˚C: to 4.0 mL of the nominal 1.0

mg/mL sample solution, 2.0 mL of 1.0 M NaOH was added, shaken, and allowed to react for 15 minutes. Then 2.0 mL of 1.2 M HCl and 10.00 mL of 0.0050 M triiodide was added, swirled to mix, and allowed to react for an additional 15 minutes, during which time the dark yellow color from the triiodide faded. 125 μL of the pale yellow test solution was pipetted onto each of the twelve squares of the test card (in the lab at MTRH, a disposable 1 mL plastic pipet was used to place 4 drops of the sample on each sample loading area). Using the pipet tip, the solution meniscus was drawn across all 5 subsections of the square to cover it completely. The card was left on a flat surface and gently moved back and forth about 1 cm at a rate of 2 Hz for 3 minutes to help mix the contents of each square. Then, a picture of the paper test card was taken in a lightbox and the image was read by visual comparison to standard images. The sample preparation procedure allows the units to be converted directly from "mg/mL" to "% of labeled amount" (e.g., if a sample matches the 0.90 mg/mL standard image, the sample contains 90% of the labeled antibiotic amount).

Internal validation. Analyst 1 prepared the stock samples for analysis on both HPLC and the test card. Analyst 1 performed only the HPLC analysis and kept the results a secret from Analyst 2. Analyst 2 performed the test card analysis and reported the results to Analyst 3, after which time Analyst 1 unblinded the HPLC results.

Powder X-ray diffraction of insoluble material isolated from amoxicillin capsule. A white powder was recovered from one of the suspect samples by extensive washing of the powder on a Hirsch funnel (water, 1 M NaOH, 0.5 M HCl, methanol, acetonitrile, acetone, toluene, 1-propanol, and hexane). After the hexane wash, the powder was dried in a 100˚C oven for 1 hour and then used for XRD and IR. Data were recorded as a

series of 360 degree phi rotation photos at 250 K using monochromated Cu radiation with an APEX-II area detector operating in 1Kx1K mode at 15 cm specimen to detector distance, yielding an effective scan resolution of 0.01 deg. Data to 30 degree in 2-theta were recorded for 60 seconds per rotation frame and from 30 to 80 degree at 120 seconds per frame. The images were composited and intensities integrated from the composite image using the APEX-3 suite of software.

IR of insoluble material isolated from amoxicillin capsule. IR was obtained by spreading the sample on an ATR plate. 16 scans at 4 cm^{-1} resolution were acquired and the air background was subtracted. The baseline was corrected using a linear fit; the data are unsmoothed.

RESULTS

Preliminary examination of Kenyan beta-lactam pharmaceuticals. None of the published studies about amoxicillin and ampicillin quality (listed in Table S1) reported the actual API contents of their samples[11,12,13,14,15,16,17,18,19] and this information was needed to develop the proper quantitative analysis range for the paper test card. In 2013-2014, secret shoppers collected patient dosage units of amoxicillin, amoxicillin/clavulanate combination capsules, and ampicillin in western Kenya (see "Pharmaceutical sample collection in Kenya" in the Methods section). Of 189 samples analyzed by HPLC, 46 (24%) samples failed the USP standard for API content. Of the failing samples, 32 (70%) contained 80.0-89.9% of the labeled API (Table 1), 13 (28%) contained <80% API content, and only one sample (2%) tested above 120% API content. Based on these results, the test card analysis was tuned to detect substandard products,

particularly those that are marginally substandard. This means that the card might miss overdosed products.

TABLE 1. INITIAL DETERMINATION OF THE QUALITY OF KENYAN BETA-LACTAM PHARMACEUTICALS

Pharmaceutical	Failing samples*	< 80.0%	80.0-89.9%	> 120.0%
Amoxicillin	31/128	3	28#	1#
Amoxicillin/ Clavulanate	2/54+ 14/54+	1 11	1 3	0 0
Ampicillin	1/7	0	1	0

Note: *Using USP's assay acceptance criteria of 90.0-120.0% of labeled amount of API. #From one unit, 1 capsule tested 80.0-89.9%, a second capsule tested as 90.0-120.0, and a third capsule tested as > 120.0%; since 2 out of the 3 capsules failed, we classified the unit as failing, and both categories are tallied in the table. +54 combination capsules were analyzed in total and 2 capsules failed for both amoxicillin and clavulanate content. In all, 46 out of 189 units (24%) failed.

Design of antibiotic paper analytical device (aPAD). The aPAD test card was engineered to measure API content in the 80-110% API range. A previously reported iodate titration card[8] was modified to create the aPAD, as detailed in the supporting information section S2. The main design goal was to distinguish substandard samples from good samples (e.g., below 90% API content should be reported as substandard, while ≥90% API should be reported as meets standard).

The aPAD contains three columns of four squares; each square is divided into five loading zones that hold the chemical reagents needed for a simple iodometric limit test. The loading zones are separated by thin wax lines, and the squares are delimited by thick wax lines. When the sample solution is placed on a square, the solution meniscus flows over the thin wax lines but is pinned by the thick wax lines, so reagents stored in the loading zones can dissolve and mix in the surface-bound drop. Each square in a column contains a different level of thiosulfate that detects a limited quantity of iodine, so the

column of squares performs a titration and the entire card does the titration in triplicate. A user prepares the sample as detailed in the methods section, applies 125 μL of the solution to each square of the test card, and then picks the standard image (shown in Figure 2 and Figure 3) that best matches the test card's response. The remaining features printed onto the test card are a QR code and fiducial marks, which enable automated image analysis if a picture of the card is taken with a cell phone.

Calibration of the test card. The aPAD was calibrated using amoxicillin trihydrate and ampicillin. Standard images are shown in Figure 2 and Figure 3. The calibration ranges expressed in mg/mL are slightly different for the two antibiotics because they have different formula weights.

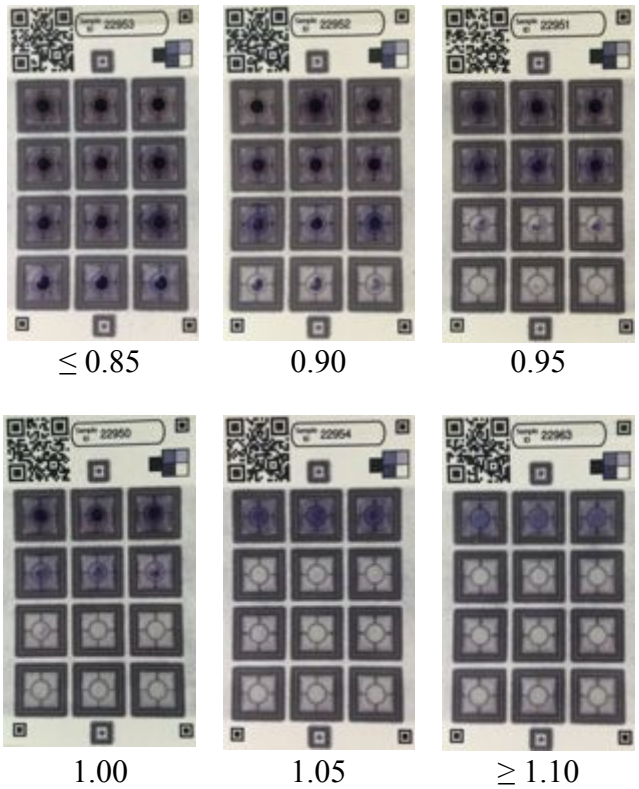


Figure 2. Amoxicillin standard images. Units are mg anhydrous amoxicillin per mL water.

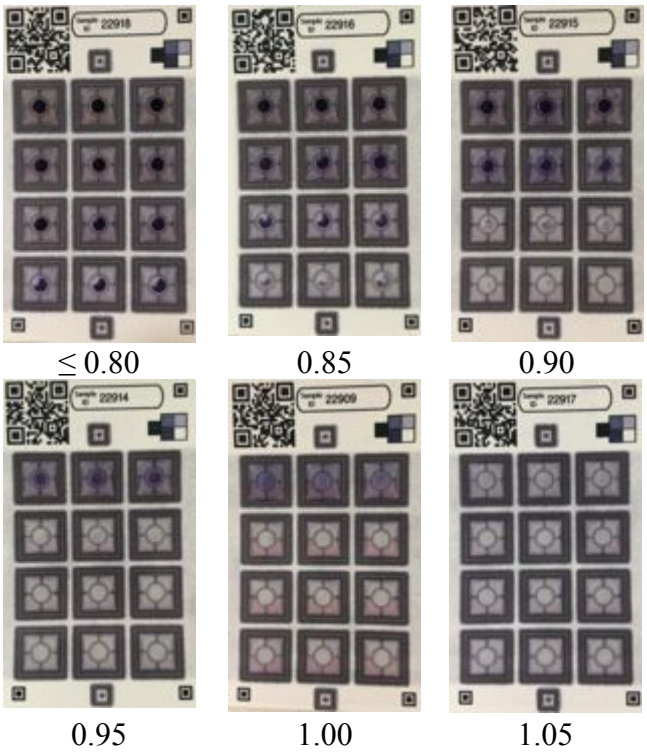


Figure 3. Ampicillin standard images. Units are mg ampicillin per mL water.

The number of blue circles and their intensities vary from one standard image to the next. The smaller the quantity of antibiotic in the sample, the more iodine is left unreacted in solution, and the more circles in each column turn blue. The cards here are run as triplicate samples, so circles in each row should have the same color intensity. Varying the concentration of antibiotic by just 0.05 mg/mL produced good visual distinction between the colors in the blue dots on the test card over the 0.80-1.10 mg/mL range.

The color in the blue dots becomes saturated for samples whose concentrations are at the ends of the range. When the card is saturated, the amoxicillin response cannot be quantified, but it can be classified as under the limit of quantification of the test card (ULOQ ≤ 0.85 mg/mL, or ≤ 85% of labeled dosage amount) or over the limit of quantification of the test card (OLOQ≥ 1.10 mg/mL, ≥ 110% of labeled dosage amount).

A similar scheme is applied to the ampicillin response to classify it as under limit of quantification (ULOQ ≤ 0.80 mg/mL, ≤ 80% of labeled dosage amount) or over limit of quantification (OLOQ ≥ 1.05 mg/mL, ≥ 105% of labeled dosage amount). Any sample that is ≤ 85% of the labeled dosage amount is classified as substandard; this includes all of the ULOQ amoxicillin samples plus those reading 0.85 mg/mL, and all of the ULOQ ampicillin samples plus those reading 0.80 mg/mL or 0.85 mg/mL. This test card design is not sensitive for detection of overdosed products, but in our experience, these make up a very small fraction of the bad quality products found in the market.

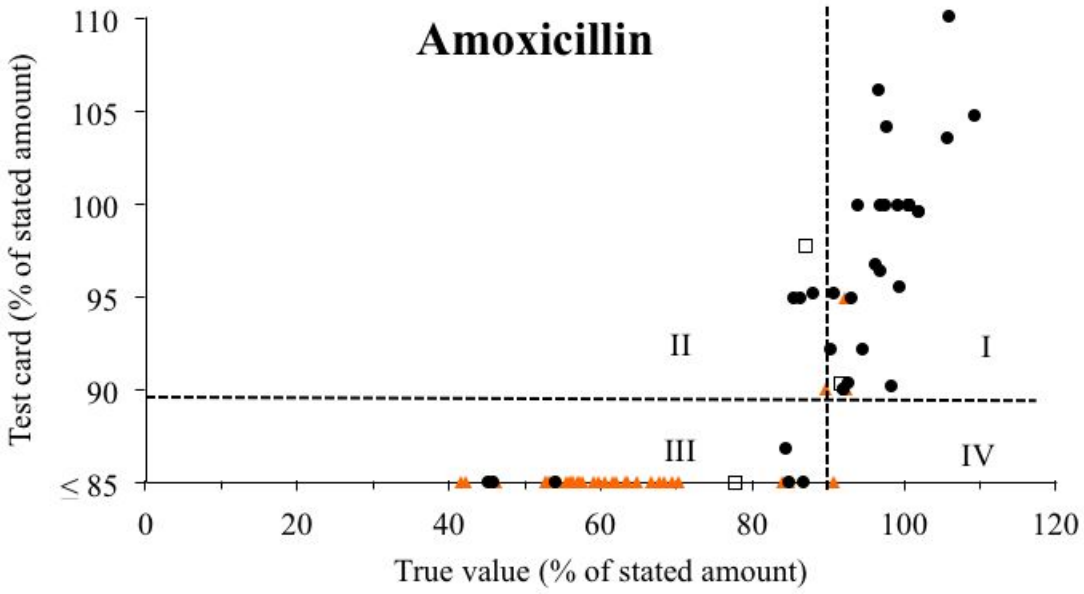
Variation of visual analysis. Since reading the test cards by visual analysis is subjective, we measured the variation that arises when different operators read the test cards (Table 2). This study was conducted on the first 22 images collected during the validation study. The true values were blinded, and the operators read the test cards independently. At the start of the study, each analyst had a different level of experience with the aPAD. Analyst 1 developed the technology, analyst 2 was an experienced reader, and analyst 3 was newly trained. Of the 22 images, 8 corresponded to under limit samples, and all analysts correctly evaluated these cards. The remaining 14 images fell into the quantitative range of the aPAD. All three analysts achieved absolute errors of 2-3%. The standard deviation of the 3 reads for each test card was calculated, and then all 14 standard deviations were averaged to determine the inter-reading precision of 0.6%. All analysts achieved similar errors in reading the test card, which means the newly trained user could interpret the test card nearly as well as the expert readers.

TABLE 2. VISUAL EVALUATION OF APAD BY THREE READERS

Analyst	1	2	3	n
Error (%)	1.7	2.0	2.5	14
Bias (%)	0.4	0.8	1.4	14

Note: All samples were amoxicillin.

Internal validation. The analytical metrics of the test card were determined through a blinded validation study. Both HPLC and the aPAD were used to analyze single capsules taken from 140 packages of amoxicillin and ampicillin pharmaceuticals that had been collected in Kenya from 2014-2016. At the time of analysis, these products were all within their expiration dates. During the HPLC analysis, not enough deficient samples (0-80% API) were identified to measure the card's ability to detect deficient samples. To increase the sample size of substandard products, some products were diluted with talc and other fillers (see “Mocked-up amoxicillin samples” in the methods section), and 12 expired substandard ampicillin medicines collected in 2012-2013 were included. The mocked-up samples and other products were relabeled for the blinded validation study, and the researchers who performed the HPLC and the aPAD assays were not aware of each other's results until the study was completed.


Amoxicillin
Test card (% of stated amount)
True value (% of stated amount)
110
105
100
95
90
≤ 85
0
20
40
60
80
100
120
I
II
III
IV

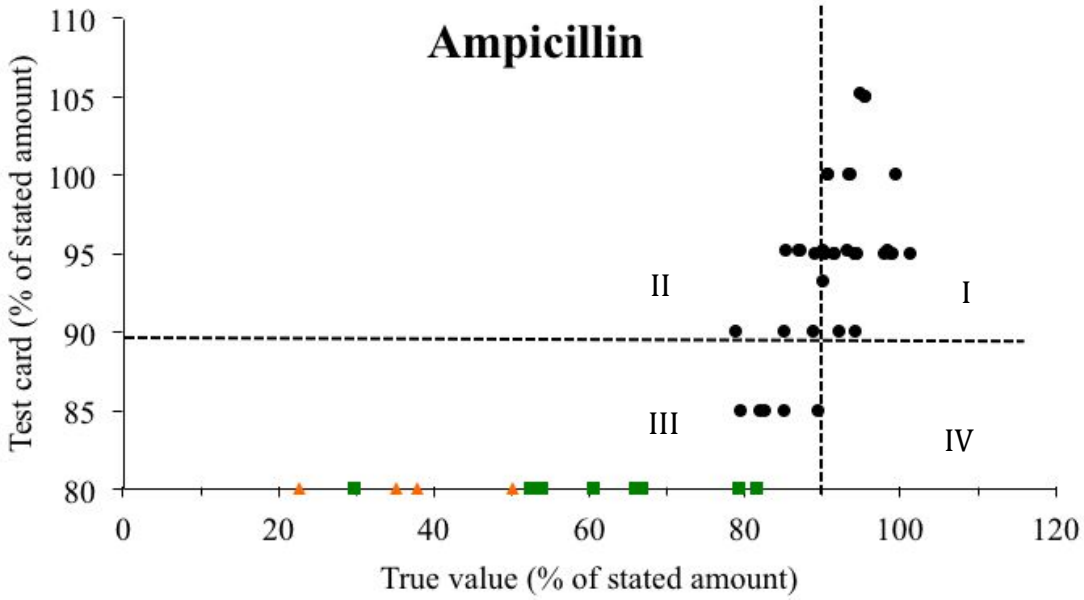

Ampicillin
Test card (% of stated amount)
True value (% of stated amount)
110
105
100
95
90
85
80
0
20
40
60
80
100
120
I
II
III
IV

Figure 4. Accuracy plot for amoxicillin (top) and ampicillin (bottom) analysis. The results are expressed as % of the stated dosage amount. Data points in quadrant I represent good quality samples reported as good quality, those in quadrant II represent bad quality samples reported as good quality, those in quadrant III represent bad quality samples reported as bad quality, and those in quadrant IV represent good quality samples reported as bad quality. The card's ability to quantify antibiotics ends at 85% for amoxicillin and 80% for ampicillin, so results at or below these thresholds are plotted on the x-axis. Samples: (●) = unexpired medicines, (■) = expired medicines, (□) = no expiry date given on package, (▲) = purposefully diluted with filler to increase the sample size of bad quality capsules.

44 of the amoxicillin samples (n=84) were real products that were intentionally diluted with fillers in lab: 35 with talc, 8 with starch, and 1 with acetaminophen. When triiodide was added to the test solutions, the starch-containing samples turned dark blue and the acetaminophen sample turned orange, and both uncharacteristic color changes flagged these 9 samples as suspicious. These 9 data points are not plotted on Figure 4, but are included in the performance metrics in Table 3 as true negatives. The remaining 40 amoxicillin samples included 37 unexpired products and 3 with no stated expiry date. Ampicillin (n=56 products) included 12 expired samples.

The performance of the test card was evaluated for detection of dosage forms that meet the regulatory requirement for API content. A test reading indicating an API content within 90-110% of the label's claimed amount was considered a good quality sample. In Figure 4, data points in quadrant I represent good quality samples reported as good quality, those in quadrant II represent bad quality samples reported as

good quality, those in quadrant III represent bad quality samples reported as bad quality, and those in quadrant IV represent good quality samples reported as bad quality.

Categorical analysis. To see if the test card can be used as a tool that rates the medicine as "good" or "bad" quality, the visual reads were grouped using the USP's 90.0% assay requirement as a limit. A "good quality" response was assigned the positive condition for the metrics in Table 3. The aPAD predicted the correct quality status for 128/140 (91%) of the ampicillin and amoxicillin samples. Based on these categorization results, 68 of the 79 bad quality samples (86%) would be detected as low quality by the test card and sent to a certified lab for additional testing, and only 1 of the 61 good quality samples (1.6%) would be sent for testing. Cohen's Kappa value is 0.83 ±0.05, meaning the aPAD and HPLC methods agree very well even when accounting for chance agreement. The test card predicted that 11 samples met the quality standard when in fact they did not (false positive rate = 11/79 = 14%), although it should be noted that these false positives were almost all marginal fails; only one of the 11 false positives had an API content below 80%. The test card predicted one sample to be substandard that actually met the API standard although again, it should be noted that this was a marginal pass at 91% API content. The analytical metrics for the two antibiotics are shown separately in Table 3.

TABLE 3. THE TEST CARD'S PERFORMANCE FOR CATEGORIZING THE QUALITY OF ANTIBIOTIC SAMPLES

		Amoxicillin n=84		Ampicilllin n=56	
		HPLC		HPLC	
		≥ 90.0% n=33	< 90.0% n=51	≥ 90.0% n=28	< 90.0% n=28
Test card	≥ 90.0 %	32	4	28	7
	< 90.0 %	1	47	0	21
Correctly categorized		94% (79/84)		88% (49/56)	
Sensitivity		97% (32/33)		100% (28/28)	
Specificity		92% (47/51)		75% (21/28)	
False positive rate		8% (4/51) (4/4 are marginal*)		25% (7/28) (6/7 are marginal*)	
False negative rate		3% (1/33)		0% (0/28)	
Cohen's Kappa		0.88 ± 0.05		0.75 ± 0.09	

* marginal quality is defined as an API content in the 85-89% range

Quantitative metrics. The test card assays the amount of antibiotic present in a sample in steps of 5% over a range of about 80-110% of the amount stated on the product's label. Of the 140 samples used in this study, 34 amoxicillin and 40 ampicillin had concentrations that fell into the quantification range of the aPAD. The analytical metrics for those 74 samples are shown in Table 4; the samples classified as under or over the card's limit of detection are not included in the calculations. The error was calculated as the arithmetic average of the absolute differences between the aPAD and HPLC response (expressed in % of stated amount).

TABLE 4. QUANTIFICATION OF AMOXICILLIN AND AMPICILLIN WITH APAD

Analyte	Amoxicillin	Ampicillin
Avg. absolute error (%)	4.4 (n=34)	5.3 (n=40)
Bias (%)	2.3 (n=37)	3.7 (n=40)
Inter-device precision (%)	0* (n=5)	2.2# (n=5)

Note: The units are expressed as % of amount found by HPLC. The error was calculated as the arithmetic average of the absolute differences between the aPAD and HPLC response (expressed in % of stated amount). *One sample with a true value of 100.6% was run 5 times and all test cards were interpreted as 100%. #One sample with a true value of 94.2% was run 5 times and the test cards were read as 95%, 95%, 95%, 95%, and 90%.

Characterizing bad quality products found during validation. During the validation study, the test card identified several packages of substandard amoxicillin capsules. The stated brand and manufacturer on the packaging of most of these samples was Caremox-500, manufactured by Shandong Shenglu Co., Ltd, with batch numbers 140604, 150601, and 150602. Repeated attempts to contact the manufacturer were unsuccessful, so we could not verify whether the product was authentic. HPLC analysis found that multiple capsules taken from these packages contained only 40-60% of the stated amoxicillin content. The product was reported immediately to the Kenyan drug regulatory authorities and the WHO RapidAlert system.

The samples prepared for HPLC analysis were very cloudy, in contrast to other brands of amoxicillin that gave solutions of low turbidity. This suggests adulteration with a filler. The identity of the filler might link this product to other products made by the same entity (which might not be the stated manufacturer) so we decided to characterize it further. An insoluble material was recovered by filtration and extensively washed with water, 1 M NaOH, 0.5 M HCl, methanol, acetonitrile, acetone, toluene, 1-propanol, and hexane. The lack of solubility of the material suggested it might be a polymer or an inorganic mineral. Infrared spectroscopy (IR) in the 4000-500 cm^{-1} range revealed no bands consistent with organic functional groups (see Figure S2) but was consistent with a layered magnesium silicate mineral, such as talc. A reference spectrum of talc is shown in Figure S3. A powder x-ray diffraction (PXRD) pattern was recorded on a representative

specimen taken from the unknown powder (Figure S4). Computer fitting of the diffraction peaks to common talc phases showed the material to be 86% triclinic talc and 14% monoclinic talc. Other common insoluble minerals (SiO_2, TiO_2 or $CaCO_3$) do not match the observed diffraction peaks. Gravimetric analysis showed that talc comprised 45% of the capsule contents by mass; the levels of talc are so high that the correct amount of amoxicillin could never have been placed into the capsules. The real manufacturer of the product is not confirmed, but regardless of who actually manufactured this amoxicillin product, it was falsified.

DISCUSSION

New tools are needed to keep substandard antibiotics from reaching patients, especially in developing countries. There are many studies that show problems with the quality of beta-lactam antibiotics in LMICs, but insufficient sampling techniques and sizes hinder tracking of regional prevalence rates. A breakdown for studies that used HPLC to assay the API content of amoxicillin and ampicillin is listed in Table S1. The prevalence of bad quality products reported in these peer-reviewed studies ranges from 0-83% for amoxicillin, and from 15-100% for ampicillin. However, the data sets available in USP's Medicines Quality Database[20] (Table S2) show that 0-13% of the amoxicillin samples and 0-4% of the ampicillin samples failed to meet the relevant pharmacopeia standards.

The large discrepancies among the prevalence rates found by different investigators indicates that the sample pool of ampicillin and amoxicillin products is heterogeneous in quality and that current sampling sizes (716 samples collected over 15

years and about 20 countries for the studies considered here) are too small to adequately capture prevalence rates of low quality products. One contributor to sample heterogeneity is the large number of brands found in many markets. While most manufacturers are diligent in applying good manufacturing practices, quality control, and quality assurance, other manufacturers lack resources to assure quality. They may cut corners to save money, or purposefully manufacture falsified products. Product quality from a manufacturer can vary over time if they change suppliers or alter manufacturing processes. Finally, even if a product meets all quality standards at time of production, it may degrade in quality after sale due to bad distribution or storage practices, which are not under the manufacturer's control. To put a positive spin on this picture, small increases in capacity for post-market testing could have a large impact in identifying low quality products for further regulatory attention. The question then becomes how to free up analytical and regulatory resources in order to conduct such testing.

The aPAD could reduce the cost of detecting substandard ampicillin and amoxicillin by a factor of 7. The "gold standard" HPLC assay used for post-market testing of beta lactam antibiotics is expensive and time consuming. A majority of instrument and analyst time is spent preparing and testing good quality samples in order to find the bad quality ones. Removing the good quality samples from the HPLC queue would free up HPLC capacity and allow a concomitant increase in the scale of sample collection. A meta-review of over 100 studies of pharmaceutical quality estimated that 10% of medicines sold in LMICs are substandard or falsified.[3] Based on this estimate, in a sample of 100 beta lactam products, on average, 10 would be of low quality. If the cost

of running an HPLC assay is $250, then the cost per detection of one bad quality product is $2,500. Based on the accuracy of the aPAD in our validation study, screening the 100 products with the aPAD at a cost of $3 per sample would result in flagging 8 out of 10 bad quality products and 2 out of 90 good quality products for further testing. These 10 products would be sent to the lab for confirmatory assay, which would still cost $2,500. Including the cost of aPAD screening, eight bad quality products would be detected at a total cost of $2,800, or $350 per detection of each bad quality product. In addition, the HPLC would not be tied up with analysis of 90 good quality samples.

The aPAD is suitable for field use. The aPAD requires quantitative sample preparation followed by degradation and analysis on the test card, which would be easy to perform in a central drug lab. In a field setting, the glassware and equipment that would be available in the analytical lab are often absent. We investigated two ways the assay can be performed without use of volumetric glassware or an analytical balance. Either the capsule's contents can be emptied into a liter bottle and 1 mL of water added per mg of antibiotic stated on the label (i.e., a 500 mg capsule diluted with 500 mL water), or a portable milligram balance and an automatic pipet can be used. A sufficiently accurate milligram balance to mass 250 mg and 500 mg antibiotic capsules for the aPAD field screening assay (Figures S2 and S3) costs about $20 USD. An automatic pipet costs about $200 USD, but as an alternative, the milligram balance can be used to mass the solutions. The reagents needed to degrade and acidify the sample (1 M NaOH, 0.0050 M triiodide, and 1.2 M HCl) can be prepared in a lab and added by weighing aliquots on the portable milligram balance or measuring them out with a volumetric pipet or automatic pipet. The back-titration part of the analysis is done using the aPAD card, so the user

does not need to prepare and standardize solutions or have access to titration equipment.[8] The reaction rate between the base-degraded amoxicillin and tri-iodide is temperature dependent, and we found that reaction temperatures between 20-25˚C give the most reliable PAD readings. For most accurate results, a user who has access to reference amoxicillin and ampicillin can generate a new set of standards to account for minor variations in temperature or triiodide concentration. The next best option is to perform the assay at 20-25C and compare the test card response to the standard images we provided, which were generated at 21˚C. The cost of materials for the entire aPAD analysis is about $0.18 USD (Table S4). Starting with capsules of ampicillin or amoxicillin, one analyst can prepare and analyze about 6 samples per hour using the aPAD.

In a small field study, the aPAD was tested at the Moi Teaching and Referral Hospital in Eldoret, Kenya using 8 samples of 500 mg amoxicillin capsules purchased in Kenya. Solutions with nominal 1.00 mg/mL concentrations of amoxicillin were prepared by emptying the contents of a single capsule (and then dropping both halves of the capsule into the water to dissolve any residual amoxicillin) in 500 mL of distilled water. Reagents for the aPAD procedure were massed on a Gemini 20 balance. The nominal 1.00 mg/mL solutions were analyzed on site by HPLC to determine the true amoxicillin content of the capsule. The results are shown in Figure S4. All samples analyzed were in the 95% - 110% API range, and the aPAD and the HPLC results had a 1.1% error and a -0.3% bias. This data illustrates the practicality of conducting the aPAD test accurately in a low resource setting.

Limitation 1: Access to confirmatory testing and other analytical resources is necessary to follow up on suspicious products. National drug regulatory agencies have

defined procedures for quarantining or recalling products. These actions can have serious economic and legal consequences for the manufacturer, impose a burden on distributors and retailers throughout the supply chain, and can reduce access to essential medications for patients. Initiating regulatory actions requires strong evidence of bad product quality, normally based on compendial HPLC methods carried out in a certified pharmaceutical analysis lab. The aPAD would not qualify as a compendial method unless it underwent careful study by the relevant pharmacopeia organizations. Thus, it can be used as a screening test but any samples flagged as substandard must undergo confirmatory assay in an accredited pharmaceutical analysis lab. In addition, finding that a medicine is substandard leaves some important questions open about why the medicine is substandard – whether it has degraded, was negligently manufactured, or has been deliberately adulterated. The answers to these questions, which are usually not provided by the compendial HPLC assay, may affect the regulatory response. For example, degradation could focus attention on packaging, distribution, or storage practices, while adulteration might result in legal actions against a manufacturer. Data from a wide range of analytical methods, ranging from simple gravimetric analysis to LC-MS or X-ray crystallography, can shed light on how a poor quality product came to be poor quality, and help to prevent repetition of the problem. Thus, the aPAD should be seen as the initial step in a coordinated process that is integrated with existing regulatory and analytical capacity, rather than a stand-alone solution.

Limitation 2: The aPAD is limited to certain medications. USP <425> has been validated for 14 beta lactam antibiotics. We have shown that both ampicillin and

amoxicillin assays can be translated to the aPAD card, and we anticipate that the other 12 antibiotics in USP<425> will also work on the card.

The USP method is only validated for monotherapies, but combination capsules are commonly used to treat resistant bacterial infections. For example, potassium clavulanate inhibits bacterial beta-lactamase enzymes, and is commonly used as a minor API in combination with amoxicillin. Clavulanate lacks the thioether group found in ampicillin and amoxicillin; under base hydrolysis conditions, the thioether is believed to degrade to a thiol; the thiol undergoes a 6 electron oxidation to a sulfoxide by triiodide in USP<425>. The reaction stoichiometry was determined by glassware titration following the USP <425> method (Table S5). After 15 minutes, one mole of degraded amoxicillin reacts with 5.7 ± 0.2 moles of triiodide, one mole of degraded ampicillin reacts with 5.7 ± 0.7 moles of triiodide, and one mole of degraded clavulanic acid reacts with 0.3 ± 0.2 mole of triiodide. Thus, USP <425> is not effective for detection of clavulanic acid, and the aPAD is not expected to give accurate detection of clavulanate in combination amoxicillin/clavulanate products.

Elevated temperatures encountered during shipping and storage may degrade medications. The test card and iodometric titration fail to accurately quantify amoxicillin samples that have been exposed to 85°C for 2 days (Table S6 and Table S7) suggesting the heat and base degradation pathways are different. The titration methods may also fail to accurately analyze substandard amoxicillin samples if they have been exposed to temperatures commonly found in the supply chain (~40°C) for long periods of time.

Limitation 3: The aPAD cannot detect some API substitutions: Of the studies listed in Table S1, only Yong performed additional testing on bad quality samples to determine the remainder of a capsule's contents. Among 20 bad quality samples, only one API substitution was identified − an ampicillin sample that contained amoxicillin.[21] Although the aPAD cannot detect substitution of amoxicillin for ampicillin because both have similar redox activity in the assay, Weaver et. al. designed a qualitative antibiotic test card that can do so with greater than 95% accuracy.[22] The other substitutions investigated in our validation (e.g., addition of starch or acetaminophen as fillers) gave unusual color results both on Weaver's card and during the aPAD sample preparation at the stage where iodine solution was added. In combination, Weaver's qualitative test card and the quantitative aPAD form a testing system that can detect both API substitution and substandard API content.

CONCLUSION

The analytical tools for measuring API content of amoxicillin and ampicillin in low resource settings are very limited.[23] In order to find bad quality samples, drug regulatory agencies currently must spend most of their scarce HPLC capacity testing samples that meet regulatory API content standards. Using the aPAD as a tool to screen out most of the good medications could focus HPLC resources on a subset of samples that are more likely to be bad quality. Because the aPAD has a high accuracy rate and is inexpensive, the cost for a combined aPAD/HPLC testing regimen would be about 7 times lower than using HPLC alone. During the validation study, the aPAD detected a brand of amoxicillin

capsules adulterated 1:1 with talc, demonstrating its ability to flag a truly falsified medication collected from the marketplace.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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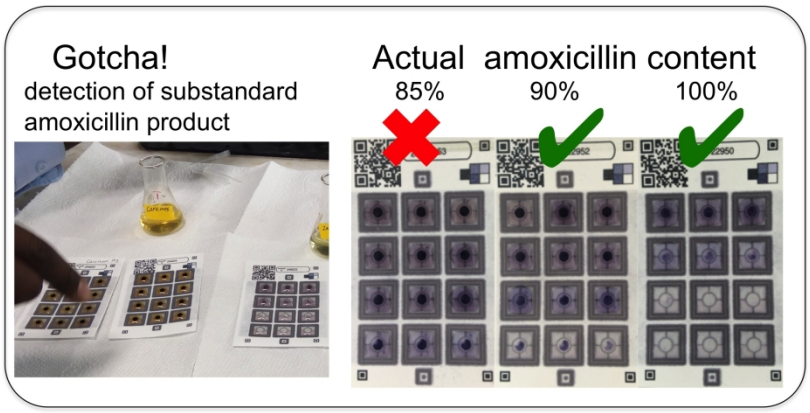

A paper analytical device can detect substandard ampicillin or amoxicillin capsules in low-resource settings.

1057x793mm (72 x 72 DPI)