Original Article

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Developing a Simple, Applicable, and Reliable Method to Produce and Standardize an Epsilometer (Etest) Strip for Antimicrobial Susceptibility and Minimum Inhibitory Concentration Evaluations

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Abstract

Microbial infections remain a major global health burden due to rising antimicrobial resistance. Rapid and accurate determination of minimum inhibitory concentration (MIC) is essential. Conventional methods like broth microdilution are limited by long turnaround times or poor precision. The Etest Epsilometer strips (Etest) provides accurate MIC readings within 16-24 hours but are imported in many countries, including Iran, facing high costs and supply issues. This study developed a simple, cost-effective method to produce Etest-like strips domestically for water-soluble antibiotics using cellulose-based substrates (K2) and a locally available adhesive (G1). Ciprofloxacin and *Escherichia coli* were used as models. Drug loading and release were quantified by validated UV-Vis spectrophotometry (λ_{max}=270 nm, R^2=0.9998, accuracy 97.21%, precision <3%). Prototype strips showed stable release after 20 minutes. Raw MIC readings from the prepared strip were calibrated against CLSI reference methods. Strips remained stable for at least two months at room temperature (25±2 °C) and 2-8 °C. This locally producible platform enables import substitution, accelerates susceptibility testing, and strengthens antimicrobial stewardship in resource-limited settings.

Keywords: Epsilometer; Etest strip; Microdilution; MIC; Impregnation; Validation; *Escherichia coli*; Ciprofloxacin.

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1. Introduction

Bacterial infections constitute a worldwide health challenge causing around 7 million fatalities annually and placing among the top 10 leading causes of death globally (1). These infections impose a clinical and financial strain due to prolonged hospitalizations, expenses related to managing affected patients and rising worries, about antimicrobial resistance (AMR) (2). In Iran 7-12% of hospital admissions are linked to health care associated infections (HAIs) and the incidence is significantly greater than in well developed countries, with most HAIs resulting from MDR Gram-negative bacteria (3, 4).

Choosing an antimicrobial agent at the correct dose is essential for attaining effective therapeutic outcomes. The minimum

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inhibitory concentration (MIC) refers to the drug concentration that inhibits visible growth of a particular bacterial isolate and this metric serves as the key parameter for guiding susceptibility-based treatment (5). The Clinical and Laboratory Standards Institute (CLSI) suggests standard methods for assessing MICs and susceptibility such, as broth macrodilution, microdilution, agar well diffusion and disk diffusion. While these reference techniques are widely employed in laboratories their use is constrained by incubation periods ranging from 24 to 48 hours the necessity for costly instruments and the inability to deliver exact quantitative MIC values through qualitative assays, like disk diffusion (6, 7).

Systematic inaccuracies on the Etest strip are removed through the creation of an exponential antibiotic gradient on a specially designed inert substrate that can be placed straight onto the agar surface. The Etest offers MIC endpoints with an accuracy of, about doubling dilution (8, 9), and allows easy visualization of the test as well as obtaining results within 16 to 24 hours by individuals possessing minimal technical expertise. Due to these benefits the Etest has become extensively utilized and acknowledged as an instrument, in clinical laboratories globally.

Nonetheless as there are no producers of strips within Iran the supply relies entirely on imports (mainly from BioMérieux- France and Liofilchem- Italy) (10). The price per unit (ranging from $ 5-15) largely depends on variations in exchange rates, which are further influenced by the international sanctions and interruptions, in the worldwide supply network. This becomes especially clear during periods of increased testing needs whether due to the surge in multi-drug resistant (MDR) pathogens or major public health crises like COVID-19 and during these times of intense demand; there are notable shortages of Etest products causing delays, in diagnostic outcomes and adversely affecting patient care management (11). Hence the aim of this project was to create an approach, for manufacturing and standardizing Etest strips tailored for water-soluble drugs with Ciprofloxacin hydrochloride serving as the model drug.

Ciprofloxacin (CIP) Hydrochloride was selected as the model antimicrobial for this research because it is a second-generation fluoroquinolone that exhibits broad-spectrum activity against *E. Coli* (a bacterium responsible for urinary tract respiratory and hospital-acquired infections) along with other Gram-negative bacteria and because of its high water solubility, strong stability, in drug formulations and well-defined PK/PD profiles (12). The bacterium *E. Coli* was employed for testing to ensure uniform conditions and facilitate comparisons, among test outcomes (13).

The particular objectives of this study was to investigate the possibility of proposing a practical , reproducible, standardizable, low-cost technique, for manufacturing Epsilometer of water soluble antibacterial drug such as ciprofloxacin in Iran using a paper-based impregnation approach.

2. Materials and Methods

Certified Ciprofloxacin Hydrochloride powder was kindly gifted by Shiraz Serum pharmaceutical company (Shiraz, Fars, Iran), *E.coli* was purchased from the Iranian Biological Resource Center (IBRC, Tehran, Iran), Mueller-Hinton Broth (MHB) and Mueller-Hinton Agar (MHA) from Merck KGaA (Darmstadt, Germany), Other than ingredient was obtained from domestic producers/suppliers.

2.1. Media and Inoculum Preparation and Standardization

Media were prepared according to manufacturer's guidelines, autoclaved (at 121 degrees Celsius for 15 minutes), and stored at 4 °C until use (14). *E.coli* was cultured according to supplier guideline and following the Lawn technique. The concentration of working standard of inoculum was calibrated with a spectrophotometer to match a 0.5 McFarland standard (~1.5×10^8 CFU/ml, at 600 nm; desired absorbance ~0.3). Both negative and positive control samples were incorporated in all microbial evaluations and tests conducted. All microbial test was done under aseptic condition and using sterile tolls.

2.2. Sterilization and Decontamination Protocols

Tools were sterilized either by applying regular autoclaving, or oven cycles. For disinfection of work areas ethanol 70% (v/v) was used. Decontamination of filter papers was done by soaking them in 70% ethanolic solution for 30 minutes, then drying in airflow cabinets at 50 °C until complete drying (15).

2.3. Preparation of Ciprofloxacin Stock and Working Solutions

A concentration of 1 mg/ml of the drug was prepared as a stock solution and used to prepare working solutions of ciprofloxacin hydrochloride. Stock solution was then serially diluted by halves in sterile polypropylene tubes to generate 10 ml working standard solutions in concentrations range of 128 to 0.0078 µg/ml, using water as diluent. The solutions were kept in containers shielded from light at temperatures ranging from 2-8 °C for no more than seven days to maintain the chemical stability of the compound (16).

2.4. The Development and Validation of UV-Vis Spectrophotometric Assays

All experiments were performed using a T80 UV/Vis spectrophotometer (PG Instruments Limited, UK) and quartz cuvettes with a path length of 1 cm. Sterile distilled water was used as a control. Each measurement was performed in triplicate. Ciprofloxacin solution from a commercial Ciprox® vial at a concentration of 5 µg/ml was analyzed by scanning from 200 nm to 400 nm. Its spectrum was compared with the Clarke's Analysis of Drugs and Poisons reference spectrum. The maximum absorption at wavelength λ_{max}=270 nm was chosen for all measurements.

2.4.1. Generation and validation of Calibration Curve

Eight typical concentration series (0.25, 0.5, 1, 2.5, 5, 10, 20, and 25 µg/ml) were prepared by serial dilution across 3 separate test days. The UV absorbance of each concentration was measured and recorded and the average value, for each concentration was calculated from the data of the three test days. A linear regression was conducted on the averaged data using Microsoft Excel program (version 2021) to develop the calibration curves and calculate the linearity, precision, accuracy and inter and intra-day variations.

2.5. Paper Substrate Screening and Optimization

Five different types of available cellulose filter paper (K1-K5) were prepared and their characteristics as a suitable paper for Etest strip were evaluated.

2.5.1. Sheet weight and Thickness

For every kind of filter paper sheets five pieces sized 1.5 × 4 cm were randomly cut and each of them individually was weighed with a balance (sensitivity=0.0001 g). The thickness of each filter papers was obtained either from the companies' official websites or by referring to the specifications printed on the packaging sheets provided by the manufacturer. The weight and thickness were presented as average ±SD.

2.5.2. Solvent Uptake Capacity

Five strip from each filter paper sheets sized 1.5 × 4 cm were randomly cut. Each strip weight and was submerged in either water, ethanol, acetone or a water-ethanol mixture (50:50 V/V) for 24 hours. After soaking, the strips were taken out. Kept in a corresponding well closed solvent saturated box for 10 minutes to allow excess solvent to be separated. The Solvent Uptake (g/cm^2) and Solvent Uptake Ratio were calculated using the following equation 1 and 2:

$$Solvent\ uptake = \overline{w}_{wet} - \overline{w}_{dry} \quad \text{(Eq. 1)}$$

$$Solvent\ uptake\ Ratio = \frac{\overline{w}_{wet} - \overline{w}_{dry}}{\overline{w}_{dry}} \times 100 \quad \text{(Eq. 2)}$$

W= average weight

2.5.3. Thermal Stability Testing of Ciprofloxacin

Ciprofloxacin concentration of 5 µg/ml was subjected to incubation at four different temperature conditions (25 °C for 24 hours 40 °C for 30 minutes 50 °C for 30 minutes and 80 °C, for 30 minutes). Upon finishing each exposure, UV spectra and absorbance of each solution was assessed and compared with fresh Ciprofloxacin concentration of 5 µg/ml as control.

2.5.4. Qualitative paper suitability evaluation

The suitability of the paper in terms of quality characteristics such as surface integrity , cut quality, mechanical durability, after impregnation and drying at 50 °C of filter papers were assessed through visual evaluation and accessibility and expense of was evaluated based on available market data employing a 5-point Likert -type ordinal scale scoring system (+++++ = Excellent, to + = poor).

2.5.5. Microbial contamination and Decontamination Efficacy test of filter paper

Three strips of K2 filter paper sheet which were cut in 1.5×4 cm pieces and subjected to ethanol solution for 30 minutes for decontamination treatment and dried at 50 °C for 30 minutes under aseptic condition together with 3 other untreated K2 filter paper strip with the same size were positioned on Mueller-Hinton agar plates inoculated with a sterile swab, a microbial suspension of *E. coli* (105 CFU/ml) by the Lawn culture method, and incubated at 35±2 °C. The growth of bacteria around each groups evaluated and compared.

2.6. Drug Loading Efficiency

Ciprofloxacin hydrochloride solutions with concentrations of 100, 200, 500 and 1000 µg/ml were prepared in test tubes. 100 µl of each concentration was loaded onto pieces of paper and dried. Then each piece was transferred to a glass tube and the drug content inside the paper was extracted using 10 ml of distilled water and vortexing (for 5 min), sonication bath (for 15 min) and centrifugation (for 10 min at 3000 RPM). Finally, the amount of ciprofloxacin released was measured using the developed UV spectrophotometric method. The extraction yield and loading efficiency were calculated.

2.7. Assessment of surface distribution Uniformity of K2 filter paper sheet for drug loading

The consistency of drug loading uniformity through all parts of K2 filter paper sheet were assessed by immersing and soaking a 5×5 cm K2 Sheet in 200 µg/ml ciprofloxacin solution followed by drying as described earlier. Then the dried sheet was divided into four equal parts and drug content of each part was extracted and analyzed using the validated UV Spectrophotometric Method described earlier. Uniformity outcomes were presented as %RSD (<5%).

2.7.1. The effect of surface area on Loading amount of ciprofloxacin by K2 paper

K2 filter paper were cut in four different sizes (0.9×5 cm, 2.5×5 cm, 3×5 cm, 5×5 cm). Each pieces were immersed and soaked in of 50 µg/ml ciprofloxacin solution for 24 hours. Then drug content of each pieces was extracted and analyzed as described earlier. correlation between surface area and drug loading amount was assessed.

2.7.2. The effect of impregnation media Concentration on Loading amount of ciprofloxacin by K2 paper

Three 5×5 cm pieces of K2 filter paper were cut. Each pieces were immersed and soaked into 20, 50, or 200 µg/ml ciprofloxacin solutions for 24 hours. Then drug content of each pieces was extracted and analyzed as described earlier. correlation between Concentration and drug loading amount was assessed.

2.8. Adhesive Selection and Characterization

Four categories of items (G1-G4) were assessed.

2.8.1. Qualitative Adhesive Suitability and Performance Assessment

Four different paper glue (G1-G4) available in country were purchased from domestic suppliers The qualitative characteristics of adhesives such as, adhesion strength, drying duration, application simplicity, adhesive leakage, accessibility in application were assessed through visual evaluation or evaluated based on available market data (availability and price was). A 5-point Likert -type ordinal scale scoring system (+++++ = Excellent, to + = poor) was used for comparison.

2.8.2. Intrinsic Antibacterial Activity and Microbial Contamination of adhesive

Adhesives were evaluated for intrinsic antibacterial activity and microbial contamination. Simply a drop of each glue were either dropped on a Mueller Hinton agar plates inoculated with a sterile swab, a microbial suspension of *E. coli* (105 CFU/ml) by the Lawn culture method or a sterile Mueller Hinton Agar plate, and incubated at 35±2 °C. The growth of bacteria around each groups evaluated and compared with positive and negative control plates.

2.9. Cumulative Release profile of ciprofloxacin from k2 paper Using a Franz Diffusion Cell

A K2 disk containing 200 µL of ciprofloxacin at 27 mg/ml concentration and an area of 5.07 cm^2 was placed between the donor and receptor compartments of a Franz Diffusion Cell filled with 30.0 ml distilled water in the receptor chamber maintained at 37±2 °C stirring at 100 RPM. At designated time , 0.5 ml of the solution was sampled and then replaced with distilled water at 37 °C. The corrected drug concentration at each time point was determined using the validated UV Spectrophotometric Method mentioned earlier.

2.10. Etest Strip construction

To prepare 10 Epsilometer of ciprofloxacin, a series of 20 ciprofloxacin concentration solutions with a log2 dilution range (0.0078-128 µg/ml) was prepared in distilled water and each one kept in dark sealable glass container separately. 40 strips (1.5×50 mm) and a base sheet (70×70 mm) of K2 filter paper were cut. All were sterilized with 70% ethanol and allowed to air dry under aseptic condition. Each one of twenty K2 strips was immersed in the ciprofloxacin solution for 24 hours. Then dried at 50 °C for 30 minutes, the other twenty strips of K2 strips served as spacers, between the drug-loaded strips. In a setting the base sheet was placed in a thin sterile plastic surface and fixed in place with G1 adhesive. The loaded K2 strips were arranged in descending concentration order with spacer strips interspersed in between on the surface of the base sheet and fixed in place with G1 adhesive and allowed to be completely dried and fixed. Finally, this assembly was cut into 3 mm-wide and 64 mm-length strips and the lower end of each was cut diagonally. All prepared strips were sealed in non-transparent containers and kept at 2-8 °C.

2.11. Determination of the Minimum Inhibitory Concentration (MIC) of ciprofloxacin for E. coli

Five CLSI-approved techniques were employed simultaneously to assess the MIC using an inoculum of 5.0×10^5 CFU/ml.

2.11.1. Macrodilution Method

Tubes containing 10 mL MHB and ciprofloxacin solutions with concentration ranging from 16-0.0078 µg/ml were inoculated with *E. coli* (5.0×10^5 CFU/ml) and incubated for 24 h at 35±2 °C. Growth in tubes were evaluated and compered. The concentration of the first clear tube (compared to control + and control – tubes) with the lowest concen-

tration that showed no growth was defined as the MIC concentration.

2.11.2. Microdilution Method

A 96 well plate was set up in triplicate rows with concentrations ranging from 16–0.0078 µg/ml. The MIC for the microdilution method was determined by incubating the inoculated plate (after inoculation) for 24 h. The first clear well (compared to control + and control – tubes) containing the lowest concentration was determined as the MIC concentration. The Minimum Bactericidal Concentration (MBC) was determined by subculturing the possible viable microbial content of all clear wells using MHA plates. The plate with the lowest concentration that showed no growth was defined as the MBC concentration.

2.11.3. Agar Well Diffusion Method

Wells were made on the surface of MHA plates previously inoculated with a microbial suspension of *E.coli* at a concentration of 5.0×10^5 CFU/ml. 100 µl of each ciprofloxacin solution at a concentration of 16 to 0.0078 µg/ml was added to each well. The plates were incubated for 24 hours at 35±2 °C, and at the end, the drug concentration in the well with the smallest visible zone of inhibition was determined as the MIC concentration.

2.11.4. Disk Diffusion Method

Ten µl of ciprofloxacin solution with different concentrations from 500 to 0.195 µg/ml was loaded onto blank discs and after complete drying, each disc was placed on the surface of the MHA plate, the entire surface of which had previously been uniformly cultured with a swab containing a microbial suspension of *E.coli* at a concentration of 5.0×10^5 CFU/ml by the Lawn culture method. The plates were incubated for 24 hours at 35±2 °C and at the end, the concentration formed by disc with the smallest visible zone of no growth was determined as the MIC concentration.

2.11.5. Determination of the MIC of ciprofloxacin for E. coli using In-House prepared Etest Strip

The strips were placed on the surface of the MHA plate, the entire surface of which had previously been uniformly cultured with a swab containing a microbial suspension of *E. coli* at a concentration of 5.0×10^5 CFU/ml by the Lawn culture method. The plates were incubated for 24 hours at 35±2 °C, and at the end, the concentration formed by the part of the strip with the smallest visible zone of no growth was determined as the MIC concentration.

2.12. Long-Term Stability Evaluation

Strips were kept up to 2 months either at 25±2 °C in darkness or placed in a refrigerator at 2-8 °C in darkness. Then , stability was assessed after 2 months.

3. Results

3.1. The Development and Validation of UV-Vis Spectrophotometric Assays

3.1.1. Determine λmax

The peak absorbance wavelength (λmax) of ciprofloxacin hydrochloride solution analyzed over the range of 200 to 400 nm was identified as 270 nm. This value was subsequently utilized for all subsequent measurements.

3.1.2. Confirmation of Drug Identity

The UV spectra of the drug solution from the brand (Ciprox®) showed a close match with its UV spectrum in the Clarke's Analysis of Drugs and Poisons reference (Figure 1) from 200 to 400 nm, both of which had a single peak at 270 nm and a shoulder peak near 323 nm, thus confirming that this solution was ciprofloxacin hydrochloride.

3.1.3. Creating and Validating A Calibration Curve (Linearity)

Applying linear regression to 8 con-

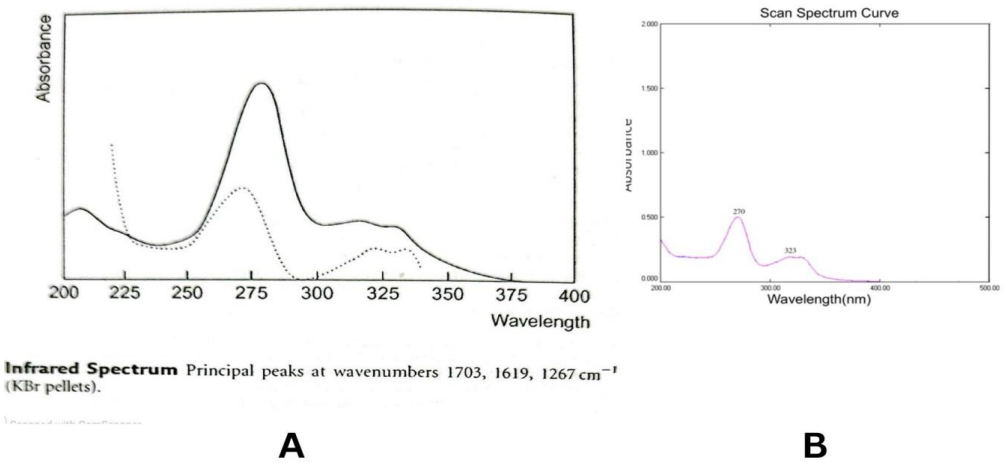

Figure 1. UV spectrum of ciprofloxacin hydrochloride in Clarke's Analysis of Drugs and Poisons reference . B : UV spectrum of ciprofloxacin hydrochloride standard solution prepared from Ciprox® brand vial in water.

centrations spanning 0.25. 25 µg/ml a linear formula was derived of y=0.0921x. 0.0106 With R^2=0.9998. Consequently the calibration curve demonstrated acceptable linearity.

3.1.4. Evaluation of Precision

The method showed a -day precision (% SEM) of 2.48% while the inter-day precision, over three days was 2.82% indicating reliable consistency of the assay.

3.1.5. Determination of Accuracy

The average percentage recovery for the assay throughout the calibration range was 97.21% indicating that the outcomes of this assay are reliable, for use.

3.2. Paper Substrate Screening and Optimization

3.2.1. Sheet weight and Thickness

Our findings showed that there is no statistically significant differences in weight variation among the five replicates evaluated for each category (K1-K5, Kruskal-Wallis, p>0.05). The % SEM was 0.61%. variation in weight, among the tested papers was significant (Welch ANOVA, p<0.001). Among the papers K1 had the least weight (0.0242 g) whereas K4 had the greatest weight (0.0484 g). The total variability within each paper was less than 5%. Paper thickness varied between 0.17 mm (K3) and 0.40 mm (K5). The test The results revealed a significant.

3.2.2. Solvent Uptake Capacity

The Kruskal-Wallis test indicated solvent absorption consistency within each paper (p>0.05) while the ANOVA tests revealed a significant differences in solvent absorption capability, between different papers (p<0.001), our results shows (Table 1) that K5 exhibited the highest water, ethanol, and 50:50 Water-Ethanol, absorption capacity while K1 showed the least. The highest absorption of acetone was observed in K3, while K1 showed the least absorption. Overall Water was chosen as the solvent to act as a vehicle for ciprofloxacin to be loaded in base paper due, to its superior solubility of ciprofloxacin in it and outstanding biocompatibility and no inherent antimicrobial activity.

3.2.3. Thermal Stability Testing of Ciprofloxacin

The UV spectra obtained from exposing the samples to different temperatures were compared and it was observed that the shapes corresponded to the UV spectra of the control

Table 1. Results of solvent uptake comparison (Water, Ethanol, Acetone, and 50:50 water-ethanol) for each paper type K1 to K5.

Solvent		Paper code				
		K5	K4	K3	K2	K1
Water	Solvent Uptake(g)	0.2936	0.0941	0.1662	0.1268	0.0934
	Solvent Uptake Ratio	9.5804	2.8462	4.3896	4.5130	4.8531
Ethanol	Solvent Uptake(g)	0.0930	0.0154	0.0538	0.0398	0.0068
	Solvent Uptake Ratio	3.6545	1.3176	2.2441	2.1363	1.2848
Acetone	Solvent Uptake(g)	0.0226	0.0021	0.0691	0.0055	0.0017
	Solvent Uptake Ratio	1.6459	1.0431	2.5301	1.1585	1.0719
50:50 water-ethanol	Solvent Uptake(g)	0.2368	0.0686	0.1157	0.1017	0.0446
	Solvent Uptake Ratio	7.7619	2.4251	3.4978	3.8946	2.8483

and that there was no change in them. Also, the drying method (50 °C/30 minutes) was implemented to create a balance between maintaining the stability of the drug and its efficacy.

3.2.4. Qualitative paper suitability evaluation

Materials from K1 to K5 that underwent processing preserved their physical forms. K2 achieved the composite score earning 22 out of 26 points; K1 had the lowest composite score, with 13, out of 26 points (refer to Table 2). K2 (Dorsan Filter Paper) was selected because of the uniformity of paper thickness , excellent water retention, quick drying capability (at 50 °C) maintaining its form when damp, ease of cutting, lightweight relative to other filter papers with strong mechanical characteristics as well, as its accessibility and affordability.

3.2.5. Microbial contamination and Decontamination Efficacy test of filter paper

Microbial decontamination was confirmed by the ineffective growth of K2 ethanol-treated samples pressed onto MHA, which did not produce any colonies after 24 hours of incubation at 37 °C.

3.3. Drug Loading Efficiency

K2 exhibited a solvent ratio of 3.30 and was capable of containing 0.0521g of ciprofloxacin within every 5×5 cm sheet. The recovery percentage, during the extraction process was 82.65% demonstrating that the drug was released more effectively compared to occasions when less efficient drug delivery techniques were applied.

3.4. Assessment of surface distribution Uniformity of K2 filter paper sheet for drug loading

A 5×5 cm K2 sheet loaded with

Table 2. Results of qualitative performance scores for evaluation criteria of paper types K1 to K5.

	Paper code	K5	K4	K3	K2	K1
Quality criteria	Proper drying ability	+++++	++++	+++++	+++++	+++++
	Maintaining the shape and uniformity of the paper surface after drying	++	+++	++++	+++++	+
	Easy and uniform cutting ability	+	+	+++	+++	++
	Ability to maintain the strength and stability of the paper during multiple processes from loading to drying on it	+	++	+++	+++	+++
	Ability to provide and access it appropriately and economically	++++++	++++++	+	++++++	++
	Total positive points	15	16	16	22	13

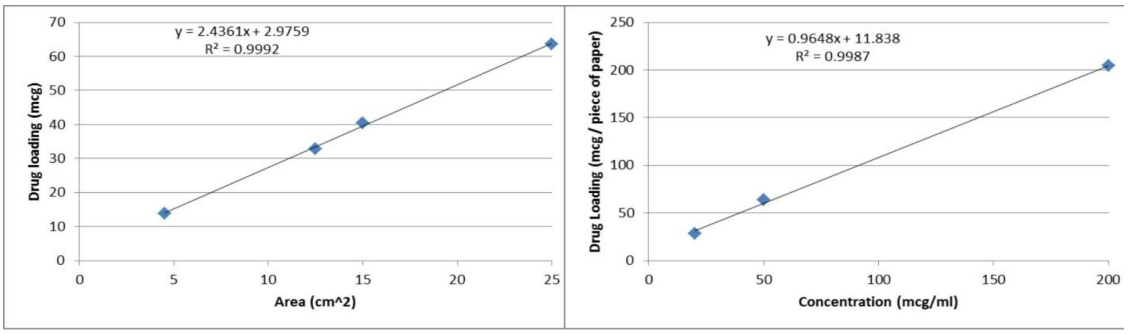

Figure 2. A: Relationship between K2 paper surface area and drug loading at a fixed concentration (50 µg/mL in all cases). B: Relationship between impregnation concentration and drug loading per segment of K2 paper with identical dimensions (all K2 papers: 5×5 cm).

20 µg/ml of Ciprofloxacin was evaluated through analysis. The mean concentrations obtained from every quadrant were 4.339 µg/ml (SD=0.058; %SEM=1.34%). The average release level of ciprofloxacin from K2 was 84.52% (with 15.48% remaining). UV spectra showed a uniform spread of ciprofloxacin, among the quadrants.

3.4.1. The effect of surface area on Loading amount of ciprofloxacin by K2 paper

A meaningful correlation (P<0.05) was identified to align with a linear rise concerning paper area and load magnitude (Figure 2-A).

3.4.2. The effect of impregnation media Concentration on Loading amount of ciprofloxacin by K2 paper

Additionally a significant association (P<0.05) indicated a linear correlation (Figure 2-B).

3.5. Adhesive Selection and Characterization

3.5.1. Qualitative Adhesive Suitability and Performance Assessment

TAll glues were sourced from suppliers. G2 adhesive was the cheapest whereas G1 was the priciest (Table 3). The composite evaluation of each adhesive ranked G1 highest with a score of 21/26 points followed by G2 (18 points) G4 (17 points) and G3 (16 points) (Table 3). G1 (Palmo Stick Glue) was selected due to its characteristics of drying (<30 seconds) strong adhesion, precise adhesive application, absence of adhesive leakage, minimal requirement for tools, during application.

3.5.2. Intrinsic Antibacterial Activity and Microbial Contamination of adhesive

No growth of any microbe was found on the MHA containing any K2-adhesives after 24 hours of incubation. K2-adhesive-K2

Table 3. Results of qualitative performance scores for evaluation criteria of adhesives G1 to G4.

	Glue code	G4	G3	G2	G1
Quality criteri	Adhesion strength	+++++	+++++	+++++	+++++
	Drying speed	+++	++	++	++++
	Ability to control the amount of use, uniformity and no leakage to the surroundings	++	+++	+++	+++++
	No need for additional tools	++++	+++	+++	+++++
	Ability to provide and access properly and economically	+++	+++	+++++	++
	Total positive points	17	16	18	21

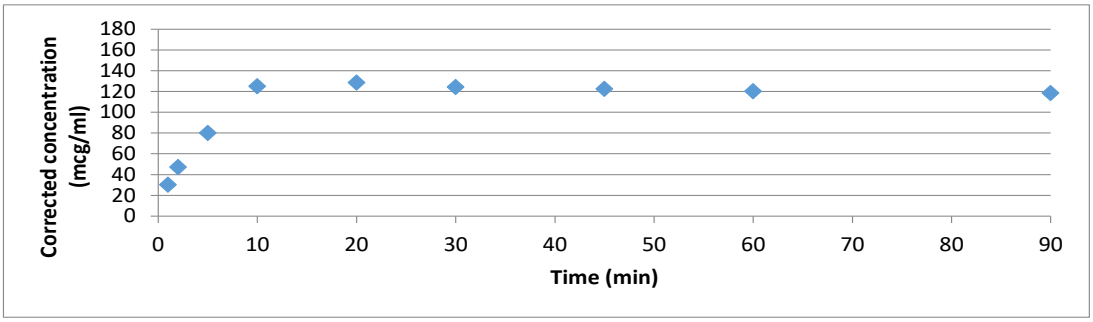

Figure 3. Corrected concentration-time curve (each point represents the mean of 3 measurements±SEM).

constructs placed on MHA coated with *E.coli* produced no inhibition zones after being incubated at 37 °C for 24 hours, thus there was no potential of interference.

3.6. Cumulative Release profile of ciprofloxacin from K2 paper using a Franz Diffusion Cell

At 20 minutes after initiation , the drug concentrations in the receptor attained their peak at 128.62 µg/ml (Figure 3). The theoretical maximum ciprofloxacin available for release (100% release) was 181.29 µg/ml so the total ciprofloxacin release from K2 was calculated to be 70.95%. The cumulative ciprofloxacin released from K2 was around 50% at 10 minutes 70%, at 20 minutes and then the cumulative percentage leveled off after 30 minutes. A 30-minute pre-conditioning or pre-diffusion period was also incorporated to the release of Ciprofloxacin, from K2.

3.7. Determination of the Minimum Inhibitory Concentration (MIC) of ciprofloxacin for E. coli

3.7.1. Macrodilution Method

MIC: 0.03125 µg/ml (first clear tube; Figure 4).

3.7.2. Microdilution Method

MIC: 0.03125 µg/ml (first clear well, triplicate; Figure 5-A). MBC: 0.0625 µg/ml (no growth on subculture; Figure 5-B).

3.7.3. Agar Well Diffusion Method

Well Diffusion were prepared to assess

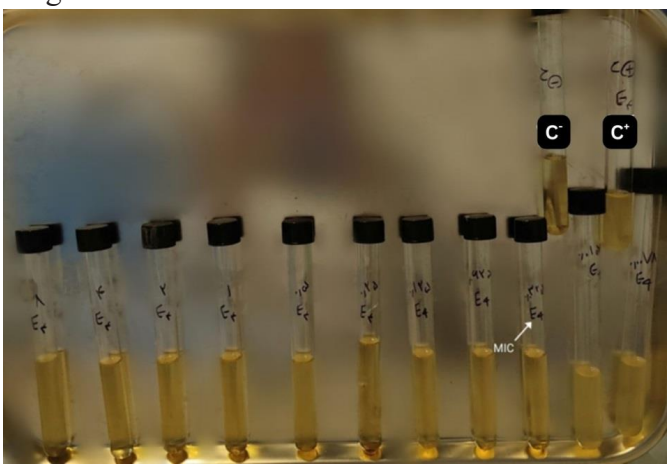

Figure 4. MIC results from broth macrodilution method. The first clear tube containing the lowest drug concentration (0.03125 µg/mL) was designated as the MIC. C+ : Positive control)Liquid culture medium inoculated with *Escherichia coli* microbial suspension to verify the ability of the broth medium to support growth of the microorganism).C- : Negative control)Sterile liquid culture medium without inoculation of *E. coli* microbial suspension to confirm that aseptic conditions were maintained throughout the procedure and to rule out contamination).

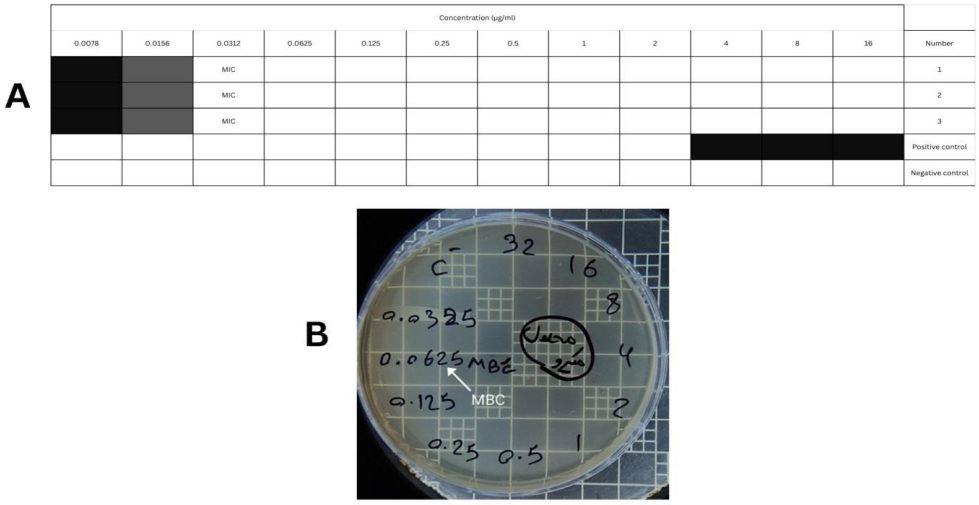

Figure 5. A: Schematic representation of a sterile U-bottom 96-well plate and MIC results from broth microdilution method. The first clear wells containing the lowest drug concentration (0.03125 µg/mL) were designated as the MIC. C+ : Positive control)Liquid culture medium inoculated with *Escherichia coli* microbial suspension to verify the ability of the broth medium to support growth of the microorganism).C- : Negative control)Sterile liquid culture medium without inoculation of *E. coli* microbial suspension to confirm that aseptic conditions were maintained throughout the procedure and to rule out contamination). B: MBC results from broth microdilution method. The sample showing no growth at the lowest drug concentration (0.0625 µg/mL) was designated as the MBC.

the inhibition zone formed by an agar medium, with drug concentrations at 0.03125 µg/ml (Figure 6). The smallest inhibition zone diameter measures 0.8 cm which corresponds to the drug concentration.

3.7.4. Disk Diffusion Method

By employing the disk diffusion technique it was observed that the minimal zone resulted from the 3.9ng drug concentration placed on the disk. For the chosen concentration (K2) measuring 1×1 cm the absorption

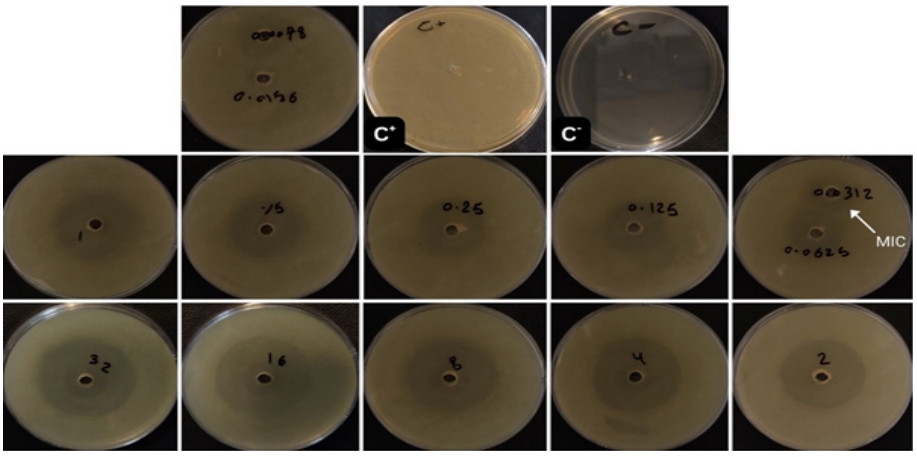

Figure 6. MIC results from agar well diffusion method. The well with the smallest inhibition zone (zone diameter 0.8 cm) containing 0.03125 µg/mL of the drug was designated as the MIC. C+ : Positive control (culture medium inoculated with *Escherichia coli* microbial suspension to verify the ability of the broth medium to support growth of the microorganism).C- : Negative control(culture medium without inoculation of *E. coli* microbial suspension to confirm that aseptic conditions were maintained throughout the procedure and to rule out contamination).

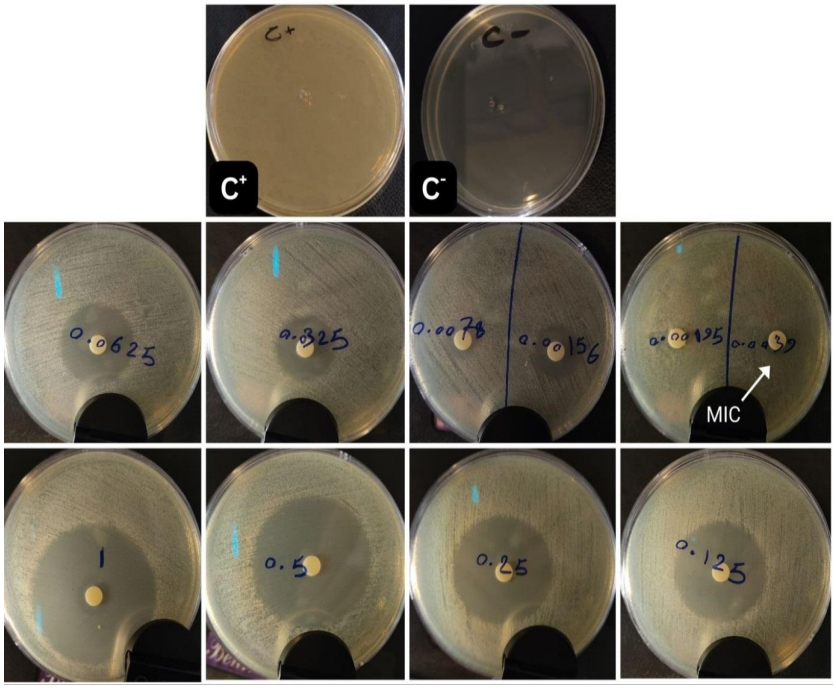

Figure 7. MIC results from disk diffusion method. The disk with the smallest inhibition zone (zone diameter 0.8 cm) containing 0.0375 µg/mL of the drug was designated as the MIC.C+ : Positive control (Disk without ciprofloxacin hydrochloride placed on culture medium inoculated with *E. coli* microbial suspension using a swab by the lawn culture technique, in order to evaluate the ability of the culture medium to support microorganism growth and the inability of the disk to inhibit microorganism growth). C- : Negative control (Disk without antibiotic placed on culture medium lacking *E. coli* microbial suspension, in order to ensure compliance with aseptic conditions throughout the various stages).

recorded was 22.2µl of the drug compared to 40.2µl for the disk of identical dimensions. With a release of 37.5% of the drug the MIC estimation was determined to be, around 0.0375 µg/ml (Figure 7).

3.7.5. Determination of the MIC of ciprofloxacin for E. coli using In-House prepared Etest Strip

To determine the MIC with the prototype Etest strip, the portion of the strip that formed the smallest zone of inhibition was immersed in a concentration of 0.5 µg/ml and contained 1.32 ng of drug. The actual crude MIC was 1 µg/ml (about 75% of the total amount of drug loaded on the strip).

3.7.6. Cross-Method Comparison and Calibration

The CLSI microdilution method required a conversion factor of ×32 to align it to the Etest strip method. In order to align the Etest strip with the CLSI microdilution method, the Etest strip number for MIC has been recalibrated to reflect the conversion factor by converting the published MIC displayed on an Etest strip to a true MIC by taking the published MIC / 32* (Figure 8).

3.8. Long-Term Stability Evaluation

No alteration was observed in the appearance, flexibility or adhesion of strips kept for two months either at a temperature range of 25±2 °C and 2-8 °C. The two-month MIC determined by the this method stayed consistent at 0.03125 µg/ml. The strips are verified to remain stable under both conditions. Strips must be kept in resistant bags containing desiccant at temperatures below 2-8 °C in darkened environments following CLSI guidelines; for additional details please consult the manufacturer.

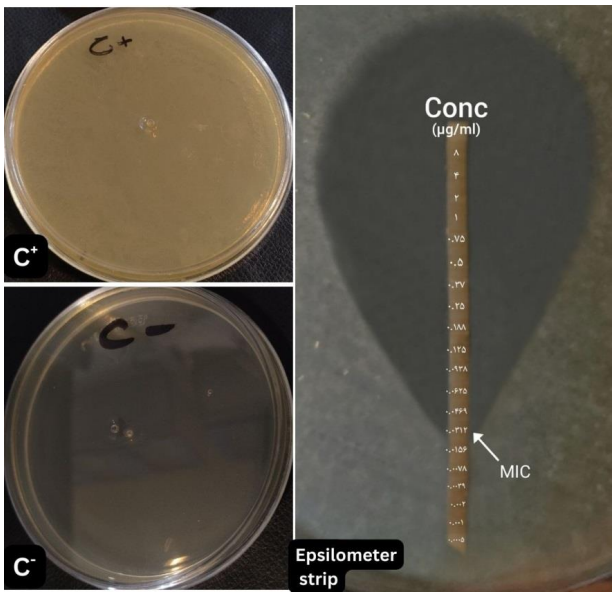

Figure 8. MIC results from the prototype Epsilpmeter strip method. The Epsilpmeter strip segment with the smallest inhibition zone (zone diameter 0.3 cm) containing 1 µg/mL of the drug was designated as the MIC (Epsilpmeter strip with recalibrated numbering aligned to conversion factors from standard microbiological MIC methods). C+ : Positive control)Agar plate inoculated with *Escherichia coli* suspension using a sterile swab by the lawn culture technique to confirm the ability of the culture medium to support microbial growth.(C-: Negative control)Uninoculated sterile medium to verify aseptic technique and rule out contamination).

4. Discussion

The multidrug-resistant *Escherichia coli* remains a challenge to delivering Health Care in both Iran and worldwide (4). Although broth microdilution—the CLSI-sanctioned reference technique for determining susceptibility—offers accurate results (the "gold standard") its practical implementation is constrained mainly due to numerous demands (such as extended incubation time, expensive reagents and instruments and the necessity, for specialized laboratory facilities) (6). The Etest, produced by the company bioMérieux overcomes many of these limitations in a sophisticated way. The Etest delivers an MIC within doubling dilution due to a standard antibiotic gradient being applied and maintained by a filtered inert medium immediate visual result interpretation and compatibility, with regular laboratory procedures (8). However the Etest is more expensive per strip than what's feasible, for most resource-limited settings and its production depends on international supply chains rendering extensive application unfeasible.

We have produced an Etest strip in Iran which can identify resistance to ciprofloxacin. This was achieved through a precisely repeatable technique, for making the Etest strip using K2 Dorsan filter paper and G1 Palmo adhesive. K2 was selected as the product due to several factors including consistent weight (SD=0.0002 g) exceptionally high water absorption (22 µl/cm²) rapid drying speed, strong durability, during cutting and handling and its low manufacturing cost. G1 demonstrated several of the benefits compared to the G1 adhesive—immediate firm bonding (under 30 seconds) complete prevention of leakage during assembly and the commercial adhesives utilized in manufacturing Etest strips were not available, in the Iranian market.

A main advantage of the strips performance design lies in the direct correlation between drug loading and both the surface area and the saturation/gelling of the paper ($r \approx 1$; $p<0.05$). Due, to this correlation gra-

dients ranging from 0.0078-128 µg/ml can be reliably produced in a way. Furthermore the strip demonstrated a stable profile with about 75% of the drug being released during the initial twenty minutes after water contacted the strip-well diffusion plates employed in this research (comparable, to conventional diffusion techniques). Crucially the drug spread uniformly across the sheet achieving a %SEM below 1.4% thereby addressing the variability concerns linked to makeshift (DIY) systems.

Relative to CLSI reference methods the MIC outcomes from this prototype (0.03125 µg/ml) matched with every detail of the proficiency testing gold standard(14); additionally the derived initial measurement (1 µg/ml), for drug concentration offered a clear ×32 calibration factor making clinical use more straightforward. Moreover the strong stability characteristics of the strip allow for a two-month shelf-life at 2-8 °C and ambient temperature without any degradation in either qualities (color, flexibility and/or adhesion) or functional potency (MIC end point). Therefore the strip is ideally suited for application in laboratories globally and, in regions where effective cold-chain management might be unavailable.

Regarding the platform as a whole it significantly lowers the expense of testing – possibly less than 5% of the price of imported Etesting – while preserving the same degree of precision and ease of use. Consequently the platform offers a scalable solution, for diagnostic laboratory operators encountering limitations in their organizations whether related to financial or supply chain issues. Potential future avenues to explore involve; confirming results with clinical *E.coli* strains and other pathogens expanding the range of antibiotic classes (including third-generation cephalosporins and carbapenems) in the collection and developing automated large-scale production with official regulatory clearance. Effective progress in these areas will not allow diagnostic labs nationwide to become independent in susceptibility testing but also significantly strengthen AMR monitoring programs and ultimately lead to better outcomes for patients, in low and middle income nations.

5. Conclusion

This study Verified, at a low expense and, through analysis a ciprofloxacin Etest strip produced solely from locally sourced filter paper and adhesive thereby removing the necessity to import materials for producing these test strips. The findings show that ciprofloxacin Etest strips made with the developed platform exhibit gradient uniformity, a swift and steady drug release, with 75% of the drug released in 20 minutes MIC endpoint values of 0.03125 µg/ml that match exactly with CLSI reference values and stability, over the 2-month testing duration at both refrigerator and room temperatures. The answer to the shortage of tools at resource-constrained facilities reliant on external suppliers, for Etest strips is to produce and supply ciprofloxacin Etest strips locally thereby significantly reducing the expense.

Future Perspectives

Scale the production of ciprofloxacin Etest strips to provincial and national levels to help meet local demand and decrease reliance on imported Etest strips.

Compare the performance of laboratory-produced ciprofloxacin strips with that of commercially sold ciprofloxacin Etest strips (such as those, from BioMérieux) in terms of accuracy, precision and consistency.

Expand the manufactured ciprofloxacin Etest strips to include other drugs soluble in water or absorbable by UV light (e.g., aminoglycosides, ß-lactams).

Manufacture multi-drug ciprofloxacin Etest strips to investigate drug synergies and antagonisms between different classes of antibiotics (e.g., aminoglycosides and β-lactams).

Investigate other methods for producing ciprofloxacin Etest strips (e.g., inkjet print-

ing) and compare the current method.

Evaluate the performance of ciprofloxacin Etest strips for a variety of clinical *E. coli* isolates and other Gram-negative pathogens against the developed ciprofloxacin Etest strips.

Automate the manufacture of ciprofloxacin Etest strips to improve consistency in the manufacture of the ciprofloxacin Etest strips.

Authors contributions

H. Montaseri (Corresponding Author) supervised and designed all stages of the study, including the conception of the idea, development of the methodology, financial support, supervision, contribution in performing all stages of experiments, including paper substrate screening, drug loading, adhesive characterization, MIC determinations, and preparing the first draft of the manuscript and critical revision of final manuscript. F. Khajeh (First Author) performed all experiments, including paper substrate screening, drug loading, adhesive characterization, MIC determinations, and wrote the original draft of the manuscript. Z. Sobhani (Second Supervisor) supervised experimental steps, reviewed and approved the manuscript, and contributed to data interpretation. A.R. Akbarizadeh collaborated in performing the microbial tests, interpretation of microbial results, and contributed to the related sections of the manuscript. M. Faraz performed statistical analyses, assisted in the evaluation and interpretation of results, and contributed to writing the results section. All authors reviewed and approved the final version of the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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