Development and Validation of a Capillary Electrophoresis Method for Quality Assessment of Metronidazole-based Drugs

Serigne Omar SARR 1,2*, EL Hadji Assane DIOP 3, Amadou DIOP 1, Adjidokpa Gloria S. ATOUN 1, Serigne Momar NDIAYE 2, Christelle Ange WAFFO TCHOUNGA 1, Rokhaya GUEYE 1, Khadidiatou THIAM 1, Thierno Mouhamed WANE 1, Aminata SARR 1, Bara NDIAYE 1, Serge RUDAZ 3, Yérim Mbagnick DIOP 1,2

1Laboratoire de Chimie Analytique et Bromatologie, Université Cheikh Anta DIOP, BP 5005 Dakar-Fann, Sénégal
2Laboratoire National de Contrôle des Médicaments, Ministère de la Santé, BP 6303 Dakar-Étoile, Sénégal
3École de pharmacie Genève-Lausanne, Université de Genève, Université de Lausanne, Genève, Suisse

* Author to whom correspondence should be addressed; E-Mail: sosarr1@yahoo.fr

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Abstract: A simple, economic and ecological capillary electrophoresis method was developed and validated for the determination of metronidazole in pharmaceutical formulations. Validation was performed by evaluating some criteria: linearity, precision, selectivity, accuracy, limits of detection and quantification. The limits of detection and quantification were 6 mg/L and 18mg/L respectively. A correlation coefficient of 0.996 was obtained in the range between 50% and 150% of the working concentration. The repeatability and intermediate precision expressed as RSD were 1.0% and 1.3% respectively. The method showed also a good accuracy with recovery percentages ranging from 94.7% to 100%. Capillary electrophoresis could be a real alternative for developing countries for the quality control of medicines in view of its economic and environmental benefits.

Keywords: Validation, Quality Control, Metronidazole, Capillary electrophoresis, Senegal, Benin.
1. Introduction

Counterfeiting is a worldwide phenomenon increasingly widespread. Particularly that of drugs is worrying because it may endanger patient health and stability of health systems. This is a growing problem, linked to the growing globalization and deregulation measures that influence the pharmaceutical market [1].

Although it is difficult to obtain accurate statistics, counterfeits account for an estimated 10% of the global medicines market. If they are found in all regions of the world, in developing countries the problem is most acute. It is thought that 25% of medicines used in developing countries are counterfeit, and in some of them, these proportions could reach 50%. This phenomenon is the cause of about one hundred thousand (100,000) deaths per year [2]. Indeed, the regular use of counterfeit or substandard medicines can lead to therapeutic failure or drug resistance. In some cases, it can cause death. According to WHO South-East Asia region, in 2001, 38% of 104 antimalarial drugs sold in pharmacies did not contain active ingredient [2].

This worsening situation is partly explained by the lack or inadequacy of appropriate technical and human resources suitable for the control and monitoring of medicines in these countries. Thus it is necessary to train quality control specialists but also to develop and validate simple, sensitive and financially accessible methods for quality control of drugs especially in Africa. In this context, the association for quality control and detection of counterfeit drugs (PHARMELP) developed the ECB (Capillary Electrophoresis Budget). It is an apparatus for controlling the quality of medicines based on capillary electrophoresis. This basic unit is proving to be a simple, environmentally friendly and inexpensive in comparison to the instrumental analytical systems currently available on the market.

The purpose of this study is to develop and validate a method for the determination of metronidazole. Then the proposed new method was applied to evaluate the quality of metronidazole based drugs marketed in two West African countries: Benin and Senegal. The results obtained with this proposed electrophoretic method were compared with those obtained by official liquid chromatographic method described in the American pharmacopeia 2013 (4).

![Fig. 1: Chemical structure of Metronidazole](image-url)
2. Materials and Methods

2.1. Reagents

Orthophosphoric acid 85% and HPLC grade methanol were purchased from Sharlau (Barcelona, Spain). Sodium hydroxide was purchased from Carlo erba reagents (Val de Reuil, France).

Metronidazole standard (pKa=2.6; pKa=14.4) and procaine (internal standard, pKa=9) were provided by the Senegalese National Medicines Control Laboratory (LNCM, 39 Avenue Pasteur, Dakar). Ultrapure water was prepared using Milli-Q water system (Millipore, Molsheim, France) and used to prepare the buffer and to dissolve standards.

A total of 11 available metronidazole based-drug samples were taken from the market in Benin (04) and Senegal (07) at the public, private and informal sectors.

2.2. Apparatus

In this study, the following apparatus were used:

- A capillary Electrophoresis (EC Budget) coupled with a UV detector LED 255, type of capillary: TSU UV-Transparent FS Tubing, 50µm ID, 375 µm OD.
- A Varian ProStar High Performance Liquid Chromatography (HPLC) system connected with Diode Array Detector (DAD) and equipped with Star 6.3 software was used to perform chromatographic analysis.
- A pH Meter Mettler Toledo Ion Analyzer 365.
- A Sartorius balance LA230S model.

2.3. Analytical Parameters

For electrophoretic analysis, a separation voltage of 20kV was applied to the electrophoretic system. The injection of solutions was done at 50 mbar during 10 s for an acquisition frequency of 9.5Hz. A phosphate buffer (pH=2.5) was used and UV detection at 255 nm was conducted for an analysis time of 7 min.

The chromatographic analysis was conducted using the method described in the United States Pharmacopeia [4].

2.4. Preparation of Solutions

Standard solutions of metronidazole and procaine were prepared for recovery studies (accuracy). Three levels of metronidazole concentrations were obtained by serial dilution: 1mg/mL, 2.33mg/mL, and 4.33mg/mL. In each case, the concentration of procaine standard was 1mg/ml.

For sample preparation, 3 tablets each containing 500mg of metronidazole drugs marketed in Senegal and Benin were grounded in a mortar. Then quantity of powder equivalent to 500mg of
metronidazole was weighed, and placed in a 25ml beaker with 15ml of methanol. After complete
dissolution, the solution was filled up to the mark with methanol. This solution was sonicated for 10
minutes and filtered through a 0.45μm filter cartridge.

All solutions were filtered through 0.45 μm Whatman cellulose filter grade 42® (GE Healthcare,
France).
The metronidazole content in samples analyzed was calculated using the following formula:

\[
\text{Metronidazole content (\%)} = \frac{\text{Experimental concentration found}}{\text{Theoretical concentration prepared}} \times 100
\]

2.5. Preparation of Solutions

The Fischer-test was used to compare the results of the assay by capillary electrophoresis with
those obtained by HPLC. The F-test is a statistical test comparing the precision (reliability) of two
analytical methods from the formula \( F = \frac{S_a^2}{S_b^2} \); where \( S_a \) is the largest value of standard deviation and
\( S_b \) the smallest standard deviation value for the same sample.

2.5. Validation Criteria

The validation method was carried out by evaluating the following parameters: linearity, accuracy,
precision, selectivity, limit of detection (LOD), limit of quantification (LOQ) as specified in the ICH
protocol [3].

- Linearity and range: for linearity, five solutions containing metronidazole standard on a range of
  concentrations from 50% to 150% (50mg/L, 80mg/L, 100mg/L, 120mg/L, 150mg/L) were
  injected (n = 5 repetitions). Areas ratio of metronidazole and procaine (internal standard) versus
  metronidazole concentrations were plotted and fit by simple linear regression. The LOD was
determined by serial dilution of working solution and the LOQ deduced using ICH guidelines
[3].

- Intra-day and inter-day precision studies: intra-day precision (repeatability) was performed at
  100 mg/L with n=8 repetitions. Inter-day precision (Intermediate precision) was performed at
  100 mg/L with n=8 repetitions on 3 consecutive days. The RSDs were calculated.

- Accuracy (recovery) was determined by comparing the concentrations really obtained with the
  theoretically expected concentrations. For that, the standard addition method was used at 3
different concentrations levels for metronidazole (100mg/L, 233mg/L, and 433mg/L) with n=3
  repetitions. The following formula was used to calculate the experimental concentrations and the
  metronidazole content in samples:

\[
\text{Experimental concentration} = \frac{\text{Area ratio}}{\text{Slope of calibration curve}} \times 100
\]
Metronidazole content (%) = \frac{\text{Experimental concentration found}}{\text{Theoretical concentration prepared}} \times 100

- Selectivity was determined by injecting the calibration standard (metronidazole and procaine) and samples of metronidazole using the developed method.

3. Results and Discussion

3.1. Validation Method

3.1.1. Linearity, range, limit of detection (LOD) and limit of quantification (LOQ)

The regression curve was linear within the concentration range of 50-150mg/L (Fig. 2). The equation of the regression was y = 0.0092x with a correlation coefficient (R²) of 0.996. Fig. 2 shows a linear correlation between metronidazole amount injected and the detector response.

These values are in accordance with ICH specifications [3] which consider a good linearity if R²>0.99.

The limits of detection (LOD) and quantification (LOQ) were respectively 6mg/L and 18mg/L and indicate the suitability of the method proposed for trace analysis.

3.1.2. Intra-day and inter-day precision studies

For intra-day precision an RSD of 1.3 which is lower than 2% [3] confirm that the developed method is reliable. The inter-day precision RSDs ranging found to be 1.0% confirm also the reproducibility of the method proposed.

These results are similar to those of Schaeppler et al [6] who obtained for repeatability RSD<2% but for inter-day precision a RSD<3%. Lin et al [8] obtained for repeatability an RSD of 1.3%, and for
inter-day precision RSDs ranging from 1.1% to 1.7% for Metronidazole. Hernandez et al [7] obtained RSDs between 2.7 and 5.7% for intra-day precision and between 9 and 12% for intermediate precision RSD.

3.1.3. Selectivity

Metronidazole has pKa of 2.6 and 14.4. After trying a few buffers (borate and phosphate buffers) at different pH (6.8, 6.5 and 2.5) with different internal standards (acetylsalicylic acid, procaine) we got better results with procaine as internal standard and phosphate buffer pH = 2.5. In these conditions, the elution time was around 5 minutes. The solvent used was methanol. At the initial step of the work, we tested 2 buffers at different pH and different internal standards. Better results were obtained with procaine as internal standard and phosphate buffer at pH=2.5. The influence of the voltage and current was also studied and optimized. Results were a separation voltage of 20kV, a pressure of 50 mbar, an acquisition frequency of 9.5Hz and UV detection at 255 nm.

Electrophoregrams obtained after analyzing metronidazole standard, internal standard (procaine), mixture of metronidazole sample and procaine in the described conditions are presented respectively in Figures 3, 4 and 5. These figures show distinct peaks at specific migration times corresponding specifically to the injected products.

Fig. 3 shows the metronidazole standard’s electrophoregram with a migration time of 4.43min and fig. 4 shows the procaine standard’s electrophoregram with a migration time of 3.22min.

![Fig. 3. Metronidazole standard’s electrophoregram](image)

![Fig. 4: Procaïne standard’s electrophoregram](image)
Fig. 5: Mixture of metronidazole sample and procaine standard’s electrophoregrams

Fig. 5 shows the mixture of metronidazole sample and procaine standard’s electrophoregrams with migration time of 3.40 min for procaine standard and 5.01 min for metronidazole standard.

Figs. 3, 4, 5 show the selectivity of the developed method with specific migration times for metronidazole and procaine standards. Also the good resolution of the peaks of both standard and samples show that the method proposed is selective.

3.1.4. Accuracy / recovery studies

The accuracy/recovery studies showed RSDs varying between 0.6% and 5.6% as shown in Table 1.

<table>
<thead>
<tr>
<th>Theoretical Concentration (mg/L)</th>
<th>Experimental Concentration (mg/L)</th>
<th>Mean area ratio</th>
<th>RSD (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>100</td>
<td>0.766</td>
<td>1.64</td>
<td>100</td>
</tr>
<tr>
<td>233</td>
<td>222</td>
<td>1.665</td>
<td>0.6</td>
<td>95.3</td>
</tr>
<tr>
<td>433</td>
<td>410</td>
<td>2.942</td>
<td>5.6</td>
<td>94.7</td>
</tr>
</tbody>
</table>

The accuracy studies showed RSDs between 0.6% and 5.6% with recovery percentages of 94.7% -100%. These values met ICH criteria which recommends for recovery percentages the range of 80-120% [3]. Sarr et al [5] also reported similar results in a previous analytical validation study.

These results are similar to those of Schaeppler et al [6] who obtained recovery percentages between 97 and 99%.

The proposed method gave recovery percentages better than those of Lin et al [8] who obtained 85.2% and 91.3% for Metronidazole.

Table 3 summarizes the validation results obtained.
3.2. Metronidazole Content in Samples Analyzed

The validated method was applied to the control of the quality of metronidazole drugs marketed in Benin and Senegal. Metronidazole samples were collected in 3 drug marketing sectors: private, public and informal and analyzed. The assay results and sample characteristics are summarized in Table 4.

Table 4: Assay results and samples characteristics

<table>
<thead>
<tr>
<th>Commercial name</th>
<th>Sample code</th>
<th>Batch N°</th>
<th>Place of sampling</th>
<th>Active ingredient identification</th>
<th>Content (%)</th>
<th>Specification (% (USP) [4]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flagyl (Metronidazole 500mg)</td>
<td>Métro/Ctn/6156/1</td>
<td>6156</td>
<td>Pharmacie Sainte Famille (Cotonou)</td>
<td>Presence</td>
<td>95.2%</td>
<td>[90.0%-110.0%]</td>
</tr>
<tr>
<td>Metronidazole 500mg</td>
<td>Métro/Ctn/81/2</td>
<td>81</td>
<td>Pharmacie Sainte Famille (Cotonou)</td>
<td>Presence</td>
<td>99.8%</td>
<td>[90.0%-110.0%]</td>
</tr>
<tr>
<td>Philco-Métro (Metronidazole 250mg)</td>
<td>Métro/Ctn/1301/3</td>
<td>1301006-1</td>
<td>Hospital Mémonin (Cotonou)</td>
<td>Presence</td>
<td>102.5%</td>
<td>[90.0%-110.0%]</td>
</tr>
<tr>
<td>Metronidazole Pharmaquick (Metronidazole 250mg)</td>
<td>Métro/Ctn/9452/4</td>
<td>945200</td>
<td>Informal market, Cotouo</td>
<td>Presence</td>
<td>98.2%</td>
<td>[90.0%-110.0%]</td>
</tr>
<tr>
<td>Supplin (Metronidazole 250mg)</td>
<td>Métro/Dkr/B0416/1</td>
<td>B0416901</td>
<td>Pharmacie Pasteur (Dakar)</td>
<td>Presence</td>
<td>105.9%</td>
<td>[90.0%-110.0%]</td>
</tr>
<tr>
<td>Nidazol (Metronidazole 500mg)</td>
<td>Métro/Dkr/5977/2</td>
<td>5977</td>
<td>Pharmacie Bourguiba (Dakar)</td>
<td>Presence</td>
<td>93.6%</td>
<td>[90.0%-110.0%]</td>
</tr>
<tr>
<td>Metronidazole Bailly-Creat (Métronidazole 500mg)</td>
<td>Métro/Dkr/87/3</td>
<td>87</td>
<td>Pharmacie Gambetta (Dakar)</td>
<td>Presence</td>
<td>101.2%</td>
<td>[90.0%-110.0%]</td>
</tr>
<tr>
<td>Metronidazole Winthrop (Métronidazole 500mg)</td>
<td>Métro/Dkr/5013/4</td>
<td>5013</td>
<td>Pharmacie Bourguiba (Dakar)</td>
<td>Presence</td>
<td>97.2%</td>
<td>[90.0%-110.0%]</td>
</tr>
<tr>
<td>Metronidazole 250mg</td>
<td>Métro/Dkr/0408/5</td>
<td>130408</td>
<td>CHU Fann (Dakar)</td>
<td>Presence</td>
<td>99.6%</td>
<td>[90.0%-110.0%]</td>
</tr>
<tr>
<td>Metronidazole 250mg</td>
<td>Métro/Dkr/1304/6</td>
<td>130403</td>
<td>Hôpital Aristide le Dantec (Dakar)</td>
<td>Presence</td>
<td>91.6%</td>
<td>[90.0%-110.0%]</td>
</tr>
<tr>
<td>Metronidazole 250mg</td>
<td>Métro/Dkr/0404/7</td>
<td>130404</td>
<td>Keur Sérigne Bi (Dakar, Informal market)</td>
<td>Presence</td>
<td>100.9%</td>
<td>[90.0%-110.0%]</td>
</tr>
</tbody>
</table>
All the samples analyzed were compliant with USP specifications [4]. The assay results showed amounts of metronidazole ranging between 91.6% and 105.9%.

Surprising results obtained in the informal market with two samples (sample code Métro/Dkar/04047 and Métro/Ctn/9452/4) could be explained by a diversion of stocks from the official market to the illicit market. Also, drugs analyzed were representative sampling specialties and generics available in both target countries.

Also similar results were obtained by Diop et al [9] on the quality control of acetylsalicylic acid and paracetamol used in Senegal; the identification and determination of the active ingredients showed no irregularity for all samples tested. This result also differs from Diop et al [10] on the quality control of cotrimoxazole medicines used in Senegal. They obtained 35.6% of non-compliant samples of the 118 analyzed.

3.3. Results of the comparison test between the ECB and HPLC methods

In order to confirm the results obtained with this new method developed by ECB, 5 metronidazole-based drugs were also assayed by an official chromatographic method described in the US Pharmacopoeia [4]. These confirmations were made at Senegalese National Medicines Control Laboratory (LNCM) and showed similar results for both methods with comparable precision (FISCHER F Test). So, on the basis of the evidence, ECB appeared as accurate as HPLC for metronidazole analysis. Table 5 presents the results obtained by the two analytical methods.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>HPLC Mean content (%)</th>
<th>SD</th>
<th>RSD (%)</th>
<th>ECB Mean content (%)</th>
<th>SD</th>
<th>RSD (%)</th>
<th>Calculated F</th>
<th>Theoretical F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metro/CTN/81/2</td>
<td>100.5</td>
<td>3</td>
<td>2.99</td>
<td>99.08</td>
<td>2.25</td>
<td>2.25</td>
<td>1.8</td>
<td>9.55</td>
</tr>
<tr>
<td>Metro/CTN/1301/3</td>
<td>103.4</td>
<td>3.38</td>
<td>2.27</td>
<td>102.55</td>
<td>2.17</td>
<td>2.11</td>
<td>2.42</td>
<td>9.55</td>
</tr>
<tr>
<td>Metro/DKR/80416/1</td>
<td>105.6</td>
<td>4.26</td>
<td>4.03</td>
<td>105.86</td>
<td>3.41</td>
<td>3.22</td>
<td>1.56</td>
<td>9.55</td>
</tr>
<tr>
<td>Metro/DKR/5013/5</td>
<td>102.0</td>
<td>0.97</td>
<td>0.97</td>
<td>97.25</td>
<td>2.13</td>
<td>2.19</td>
<td>0.207</td>
<td>19.2</td>
</tr>
<tr>
<td>Metro/DKR/0404/8</td>
<td>98.5</td>
<td>1.11</td>
<td>1.13</td>
<td>100.86</td>
<td>3.82</td>
<td>3.79</td>
<td>0.084</td>
<td>19.2</td>
</tr>
</tbody>
</table>

A similar result was obtained by Pajchel et al [11] while comparing the 2 combinations of amoxicillin/clavulanic acid and amoxicillin/sulbactam by HPLC and capillary electrophoresis.

4. Conclusion

The circulation of counterfeit or substandard drugs is a real public health problem in developing countries. WHO states that nearly 100,000 deaths per year in Africa are related to the trade of counterfeit
drugs. The fight against counterfeit medicines requires controlling each batch entering the country at official laboratories. The results obtained show that the developed method is sufficiently faithful (accurate), precise and selective to be used extensively. It could be of major interest for pharmaceutical companies, medicines control laboratories, public and university structures because of its simplicity, ecological character, and cheapness. Also, the simplicity of the procedure and the short analysis time (approximately 5 min) in comparison with previous methods should allow this method to be a useful tool for the routine analysis of Metronidazole. ECB could be a real alternative in developing countries for the quality control of drugs in order to protect population health.

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References


