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Validation of the HPLC method for the quantitative determination of sodium ampicillin for routine laboratory use

Gilbert TEUBOUBE [1,*], Emmanuel NNANGA NGA [2], Joseph GOUPAYO [3], Pierre Robert ABANDA EVA [4] and Berthe Léticia NGOBO EBENDE [1]

[1] *Research and Doctoral Training Center University of Yaoundé 1, Cameroon.*
[2] *Department of Galenic Pharmacy and Pharmaceutical Legislation of the Faculty of Medicine and Biomedical Sciences - University of Yaoundé 1, Cameroon.*
[3] *Department of Pharmacognosy and Pharmaceutical chemistry of the Faculty of Medicine and Biomedical Sciences - University of Yaoundé 1, Cameroon.*
[4] *Postgraduate training unit for health sciences- University of Douala, Cameroon.*

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Abstract

This study aims to validate a method for the quantification of sodium ampicillin by high-performance liquid chromatography (HPLC) for routine use in quality control laboratories. Validation was performed according to the recommendations of the International Conference on Harmonization (ICH Q2(R1)). The evaluated criteria included specificity, linearity, accuracy, and repeatability. Analyses were carried out using an Agilent Infinity 1260 HPLC system equipped with a DAD detector and a Microsorb-MV 100-5 C8 column (250 mm × 4.6 mm, 5 µm). The mobile phase consisted of a mixture of ethanol and ultrapure water (40:60, v/v) in isocratic mode, with a flow rate of 0.6 mL/min, an injection volume of 10 µL, and detection at 210 nm. The concentration range studied was from 60% to 140% (0.12 to 0.28 mg/mL) for both the standard and the reconstituted pharmaceutical form.

The method was specific; no interfering peaks were observed at the retention time of ampicillin (4.3 min). Linearity was excellent (R^2 = 0.9991 for the standard). The mean recovery percentages were 100.07% (standard) and 100.11% (reconstituted form), both within the acceptance range (80–120%). Repeatability yielded coefficients of variation (CV) of 0.13% for the standard and 0.28% for the reconstituted form at the target concentration (0.2 mg/mL), well below the 5% limit.

The developed HPLC method is specific, linear, accurate, and reliable. It can be used as a routine method for the assay of sodium ampicillin in injectable pharmaceutical forms in laboratory settings.

Keywords: Sodium Ampicillin; HPLC; Method Validation; Routine Analysis; Quality Control

1. Introduction

In the pharmaceutical industry, the validation of analytical methods is essential to ensure the quality and safety of medicines by guaranteeing the accuracy of active ingredient assays [1,2]. In summary, method validation is a crucial step that ensures the credibility of results obtained by analytical laboratories [3]. Quality control is a regulatory requirement, and its implementation is governed by regulatory texts, including pharmacopoeias [1,2] and guidelines from the International Conference on Harmonization (ICH) [4,5]. During method validation in the laboratory, it is necessary to verify whether the analytical method is appropriate for the analyte or compound being measured,

* Corresponding author: Gilbert TEUBOUBE

according to various criteria [6]. Analytical method validation, which is used for regulatory purposes, requires a preliminary framework to define parameters such as sample preparation, the technique to be used, its parameters, the environment, expected results, acceptable uncertainty, and deadlines [4,5].

2. Materials and methods

2.1. Experimental Designs

The number of validation runs was set to 3, assuming that one run is equivalent to one day [4,5]. For the validation experimental design, the number of replicates was set to 3, and the number of concentration levels was set to 5: 60%, 80%, 100%, 120%, and 140% of the active pharmaceutical ingredient content of the pharmaceutical product used to perform the validation [6].

All glassware used was Class A borosilicate, and all equipment was qualified [1,2].

2.2. Chemicals and Reagents

The ampicillin standard was supplied by EQDM and is reported to have a purity of over 99% [7–9]. All solvents used were HPLC grade: HPLC-grade ethanol (Merck), ultrapure water obtained from distilled water purified by the water purifier [3,10,11].

Instrumentation

Mettler Toledo analytical balance; BRANSON ultrasonic bath; SARTORIUS ARIUM MINI water purifier; HPLC system with an Agilent DAD detector [12–16].

2.3. HPLC Equipment

The active ingredient was quantified using an Agilent Infinity 1260 High-Performance Liquid Chromatography (HPLC) system equipped with an automatic injector with a 1 µL–100 µL injection loop and a four-channel (A, B, C, and D) quaternary pump [10]. Quantitative analysis of the active ingredient was performed using a DAD detector coupled to the chromatographic system [13].

2.4. Chromatographic Conditions

All analyses were performed at an ambient temperature of 25°C under isocratic conditions, with a mobile phase consisting of HPLC-grade ethanol and ultrapure water (40:60, v/v) [7,17]. Chromatographic separation was performed on a Microsorb-MV 100-5 C8 reverse-phase column (250 mm × 4.6 mm internal diameter, 5 µm particle size), with an injection volume of 10 µl [12,13]. Detection was performed at a fixed wavelength of 210 nm, corresponding to the absorption maximum of active pharmaceutical ingredient, and elution was carried out at a flow rate of 0.6 mL/min [7,10].

2.5. Preparation of Solutions

Mobile phase solvent: water + ethanol 60:40 (v/v)) [7]; Standard solution: Weigh a mass equivalent to 25 mg of standard ampicillin into a 25-mL flask, add 15 mL of ultrapure water, agitate using an ultrasonic bath for 5 minutes to completely dissolve the powder, and make up to volume with the same mobile phase. Take 2 mL of this solution and dilute to volume with the mobile phase [3,7]; Sample solution: Weigh a sample mass equivalent to 25 mg of ampicillin into a 25-mL flask, add 15 mL of ultrapure water, agitate using an ultrasonic bath for 5 minutes to completely dissolve the powder, and make up to volume with the same solvent. Take 2 mL of this solution in 10 mL and dilute to volume with the mobile phase [3,7,18,19].

2.5.1. Preparation of standard solutions (active ingredient)

In 25-mL volumetric flasks, prepare three sets (n=3) of five concentrations (k=5) ranging from 60% to 140% of the active ingredient alone [4,5]. The active ingredient standard solutions are prepared by dissolving test samples of the product in the appropriate amounts of diluent according to the desired concentration (Table 1), and are placed in an ultrasonicator for 15 minutes to homogenize them [7]. Next, using a syringe, we make up the volume to be prepared to the mark on the vials, then, using a syringe filter, we fill the vials and place them in the rack, which is then loaded into the HPLC instrument [10,12].

Table 1 Standard ampicillin test dose

Concentration en %	Volume of stock solution (µl)	Dilution volume (ml)	Concentration (mg/ml)
60	600	5	0.12
	600	5	
	600	5	
80	800	5	0.16
	800	5	
	800	5	
100	1000	5	0.20
	1000	5	
	1000	5	
120	1200	5	0.24
	1200	5	
	1200	5	
140	1400	5	0.28
	1400	5	
	1400	5	

2.5.2. Preparation of the reconstituted dosage form

The reconstituted dosage form was prepared using the same experimental methodology as for the standards [7]. The reconstituted dosage form was prepared using the same experimental methodology as for the standards [4,6].

3. Method validation

Validation is based on a number of criteria, such as specificity/selectivity, linearity, precision, and accuracy, in accordance with the guidelines of the International Conference on Harmonization (ICH) Q2(R1) (ICH 2005-b, ICH 1996) [4,5], resulting in analytical methods capable of providing reliable results [3,6].

3.1. Specificity (or selectivity)

3.1.1. Procedure

The procedure involves verifying the absence of any interference with the retention time of ampicillin separated by the HPLC method [12,13]. The selectivity of the method was verified by separately injecting the blank, the standard (STD), and the reconstituted pharmaceutical form, then comparing the typical chromatograms obtained by analyzing, on the one hand, a blank solution, and on the other hand, the STD and reconstituted pharmaceutical form solutions (the ampicillin powder contains no excipients) [7].

3.1.2. Acceptance Criteria

The analytical method is considered specific and selective if: The active pharmaceutical ingredient (API) control and the test sample exhibit the same chromatogram profile, there is no interference between the API and the blank, and the peaks can be identified (by comparing retention times), the specificity (or selectivity) test is based on the preparation of: diluent (blank), the standard solution (active ingredient alone), and the reconstituted dosage form solution [4,6].

3.2. Linearity

3.2.1. Procedure

The study was conducted: on the standard (API alone) = standard range, and on the reconstituted dosage form = product range [7]. Five concentrations spanning the study range (60% to 140%) were prepared [4]. Three sets of measurements were taken at five concentrations (0.12 mg/mL; 0.16 mg/mL; 0.20 mg/mL; 0.24 mg/mL; and 0.28 mg/mL) over three consecutive days [5]. Each solution was injected three times in a volume of 10 µL [10,12]. Before injecting the solutions, the column was equilibrated for at least 30 min with the mobile phase [13]. The peak areas of ampicillin were plotted against concentrations [3]. The results were evaluated using the least squares method to calculate the regression parameters (calibration equations and coefficients of determination) [6].

3.2.2. Data processing

Based on the experimental values, regression lines are plotted for the standard and the reconstituted sample, and the regression parameters are determined: the line Y = a*x + b; the slope a; the y-intercept b; and the coefficient of determination R^2 [3,4].

3.3. Accuracy

Procedure

Accuracy is determined from the data of the linearity study of the standard range and the test sample range, using statistical tests; for each concentration, three measurements of the dependent variables are taken [4,6]. It will be evaluated over a concentration range equal to 60, 80, 100, 120, and 140% of the theoretical active pharmaceutical ingredient content [5]. Accuracy is assessed by comparing the experimental value to the reference value through an overlap, or by demonstrating that the theoretical value lies within the 95% confidence interval of the experimental value [3].

3.3.1. Recovery

Accuracy can be quantified using a bias that expresses the difference between the measured mean and the true value [4].

Calculation of the recovered quantity:

The calibration curve is given by: regression line, calibration area =a *x + b

$$Recovered\ quantity = \frac{(Sample\ peak\ area - b)}{a}$$

Recovery percentage:

$$\%Recovery = \frac{(Recovered\ quantity \times 100)}{Introduced\ quantity}$$

3.3.2. Acceptance criteria

The method is considered accurate if all individual biases and the mean bias (mean recovery values) fall within the specified range (90% to 110%) [6].

3.5. Precision

- Repeatability (intra-day; n=6)
- Intermediate precision (inter-day)

Procedure

Perform at least 3 series of 6 theoretical concentration (100%) weighing on the reconstituted dosage form, at a rate of one series per day [4,5]. The 100% concentration solutions were prepared within the same group (ICH 2005-b), by the same operator, and under the same experimental conditions [4,20]. Each solution is injected 6 times [10]. Intermediate precision was evaluated by analyzing the results obtained over three consecutive days [4]. In practice, the reproducibility of the API in the control solution is assessed over three consecutive days using the same operator and

the same instrument [5]. Repeatability was determined by calculating the mean overlap and coefficients of variation (CV) across the 6 injections of two 100% concentration solutions [3,6].

4. Results and discussion

4.1. Method Validation Criteria

4.1.1. Specificity

We demonstrated that the analytical method developed for ampicillin was selective [4,5]. This selectivity was verified by first injecting the blank alone, the matrix alone, and the standard alone [7]. No interference was observed between the blank, which had a retention time of 2.7 min (Figure 1), and the analyte peak at 4.3 min (Figure 2) [12,13]. Furthermore, the retention time was not affected by the presence of the blank [1,2].

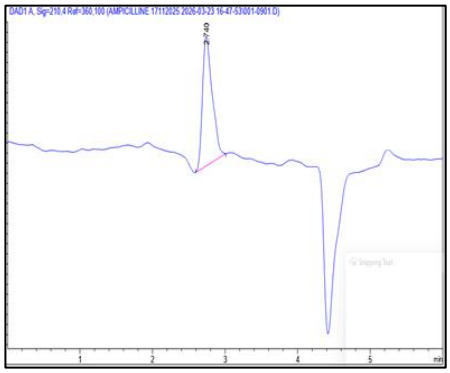

Figure 1 Chromatogram obtained by HPLC of the blank solution

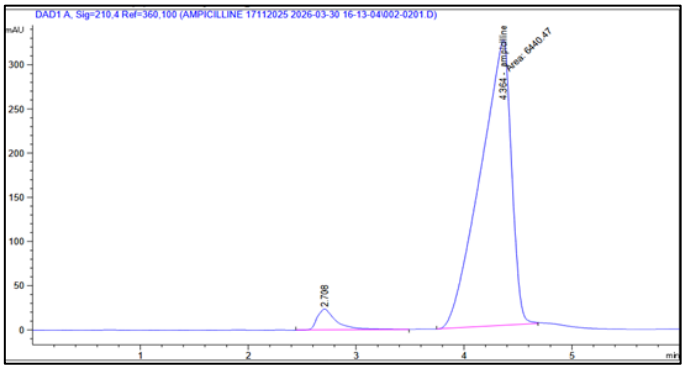

Figure 2 HPLC chromatogram of a sample solution

Based on the retention times (Figure 2) in this chromatogram, it can be seen that there is no interference with the active ingredient.

4.2. Linearity

The linearity of the methods for analyzing ampicillin was evaluated [3,4]. This linearity was determined by assessing the relationship between the calculated concentration (validation standard) and the added concentration (calibration standard) [5]. The method is linear, as illustrated in Figure 3 [7]. Indeed, the tolerance limits for each concentration level fall within the acceptance limits [6]. The correlation coefficient (R^2 = 0.9991) is close to 1.00 [3]. Other authors have also demonstrated the linearity of the analytical methods; notably, Tie et al. (2018) demonstrated the linearity of HPLC methods for the analysis of ampicillin [10].

Table 2 Peak area values as a function of concentration using the standard

Concentrations	Surface peak area
0.1248	4062.8
0.1664	5411.4
0.208	6644.2
0.2496	8154.2
0.2912	9369.8

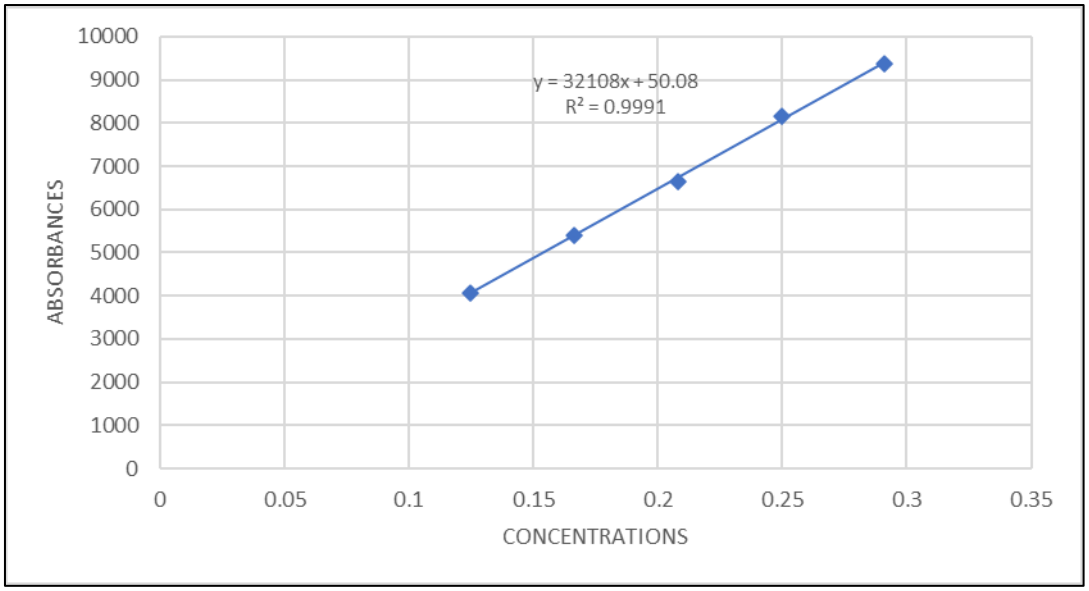

Figure 3 Calibration curve for the standard (active ingredient alone)

Table 3 Peak area values as a function of concentration using the Reconstituted dosage Form

Concentrations	Surface peak area
0.1219	4522.4
0.1625	6023.2
0.2032	7660.3
0.2438	9343.8
0.2845	10824.9

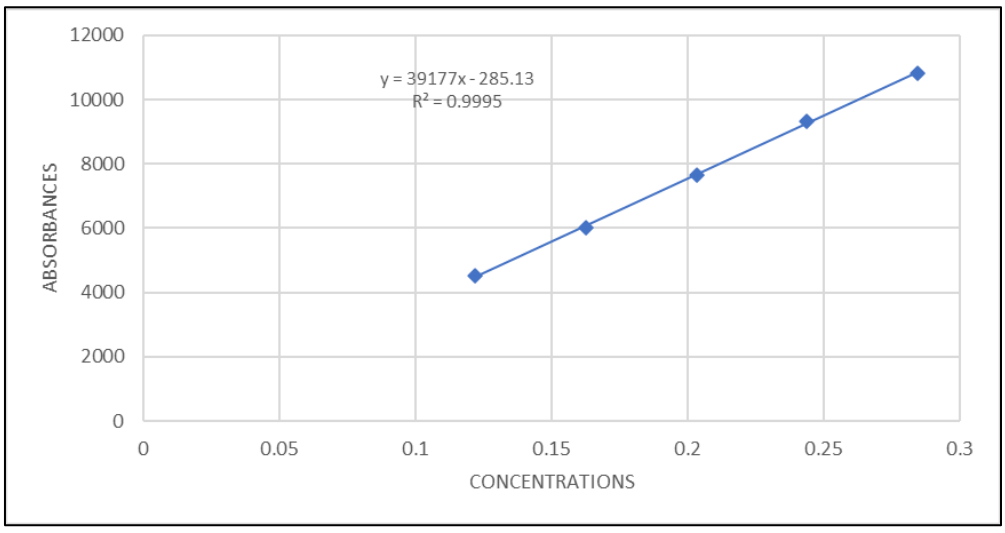

Figure 4 Calibration curve for the reconstituted dosage form

Table 4 Parameters of the linear regression equation

Parameters Studied	Standard solution	Reconstituted dosage form
Regression equation (Y= a x+ b)	Y=32108 X +50.08	Y=39177 X- 285.13
Slope (a)	32108	39177
Intercept (b)	+50.08	-285.13
Correlation coefficient (R2)	0.9991	0.9995

4.2.1. Trueness

The trueness of the method was evaluated by considering the average bias observed between the measurement series and the target concentrations [4,6]. The analyte calibration curve was then used to calculate the concentrations of the validation standard, expressed in terms of absolute bias (mg/mL) and relative bias (%) [3]. The results obtained showed that the developed method is accurate (Table 5) because the systematic error values obtained exhibit a relative bias of no more than ±5% [10].

Equation 5: Relative Error $= \frac{Conc.theo - Conc.cal}{Conc.theo} X100$

Table 5 Statistical analysis of accuracy by concentration level

Theoretical Concentrations	Areas	Calculated Concentrations	Bias (%)
0.1248	4064.6	0.1250	-0.19
	4062.8	0.1250	-0.14
	4056.7	0.1248	0.01
0.1664	5411.4	0.1670	-0.35
	5428.1	0.1675	-0.66
	5411.4	0.1670	-0.35
0.2080	6639.7	0.2052	1.33
	6644.2	0.2054	1.26
	6637.8	0.2052	1.36
0.2496	8154.2	0.2524	-1.12
	8181.1	0.2532	-1.46
	8198.6	0.2538	-1.68
0.2912	9393.1	0.2910	0.07
	9369.8	0.2903	0.32
	9348.5	0.2896	0.55
Tolerance			±5

4.2.2. Accuracy

According to the ICH, accuracy was evaluated at five concentrations covering 100% (60%, 80%, 100%, 120%, 140%) [4,5]. The results obtained during the accuracy study are shown in Tables 6 and 7 below [4] : The accuracy of the method is verified based on a maximum deviation for the dosage range, which falls within the pre-set acceptance limits of 5% [4]. The mean recovery percentage (for a total of N=5 trials) is 100.07% for the standard and 100.11% for the FPR with a confidence (risk) interval of ±5%, resulting in a mean recovery percentage range for the method of [98.64–101.68%];

This value is very satisfactory over the range of 80% to 120% of the standard concentration, meaning that the values are accurate and precise [3,6].

Equation : $R = \frac{Conc.cal}{Conc.theo} X100$

Table 6 Accuracy parameters using the standard solution

Concentration	Areas	Calculated Concentrations	Recovery
0.1248	4064.6	0.1250	100.19
	4062.8	0.1250	100.14
	4056.7	0.1248	99.99
0.1664	5411.4	0.1670	100.35
	5428.1	0.1675	100.66
	5411.4	0.1670	100.35
0.2080	6639.7	0.2052	98.67
	6644.2	0.2054	98.74
	6637.8	0.2052	98.64
0.2496	8154.2	0.2524	101.12
	8181.1	0.2532	101.46
	8198.6	0.2538	101.68
0.2912	9393.1	0.2910	99.93
	9369.8	0.2903	99.68
	9348.5	0.2896	99.45
Mean recovery (%)			100.07
Limits			80 – 120 %

Table 7 Accuracy parameters using the reconstituted dosage form

Concentrations	Areas	Calculated Concentrations	Recovery
0.1219	4551.8	0.1243	101.98
	4522.4	0.1236	101.37
	4534.5	0.1239	101.62
0.1625	6037.1	0.1614	99.32
	6023.2	0.1610	99.10
	6006.2	0.1606	98.84
0.2032	7682.8	0.2025	99.64
	7619.6	0.2009	98.87
	7660.3	0.2019	99.37
0.2438	9382.2	0.2449	100.45
	9343.8	0.2439	100.06
	9355.2	0.2442	100.18

0.280	10804.1	0.2804	100.14
	10824.9	0.2809	100.33
	10822.1	0.2809	100.31
Mean recovery (%)			100.11
Limits			80– 120 %

4.2.3. Precision (repeatability)

An assessment of the random error shows that the method is accurate [4,5]. Indeed, the repeatability and intermediate precision CVs (less than 5%) for the target concentration (100%) (Table 8) are acceptable, with maximum values of 0.12 and 0.27 [3,6].

Table 8 Determination of the coefficient of variation for the PA at a concentration of 0.2 mg/mL

Number of injections	**Areas**
1	6652.6
2	6639.7
3	6644.2
4	6637.8
5	6632.6
6	6653.3
Mean	6643.366667
Standard deviation	8.306302828
Coefficient of Variation (CV)	0.1

Table 9 Détermination du coefficient de variation pour la FPR de concentration 0.2mg/ml

Number of injections	**Areas**
1	7682.8
2	7619.6
3	7660.3
4	7642.1
5	7650.8
6	7659.6
Mean	7652.533333
Standard deviation	21.08455991
Coefficient of Variation (CV)	0.3

5. Conclusion

The results obtained in this study demonstrate that the HPLC system, equipped with a DAD detector and an autosampler, offers excellent analytical performance for the analysis of injectable ampicillin: excellent sensitivity and good linearity between 0.12 mg/mL and 0.28 mg/mL enabled the accurate quantification of the active ingredient; the %RSD of the peak area for concentrations calculated at 0.2 mg/mL was less than 2.0%, which complies with the USP

standard; the calculated recovery rates fell within the 80–120% range established by the ICH. This validated method can be applied for the assay of an injectable ampicillin dosage form.

Compliance with ethical standards

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Disclosure of conflict of interest

No reported conflicts of interest.

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