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Original Article

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# Dosage by High Performance Liquid Chromatography (HPLC) coupled with the dissolution of a combination of anti-tuberculous: a pilot study conducted in Cotonou, Benin

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## Abstract

Efficient tuberculosis treatment requires the use of good quality medicines. The present study developed a method of dosage by High Performance Liquid Chromatography (HPLC) coupled with the dissolution of a combination of fixed doses of four anti-tuberculous (Isoniazid, Pyrazinamide, Ethambutol Hydrochlorate, Rifampicine). The elaborated protocol was thereafter used to assess the quality of medicines commonly used in Benin as per the National Program for Tuberculosis Control (PNT). An analytical procedure was developed and validated by statistical tests of Cochran and Grubbs. Five lots of anti-tuberculous were randomly sampled (from India and tuberculosis screening and treatment centres in Benin). These lots were subjected to a number of tests including preliminary, disintegration, identifications and dissolution tests coupled with HPLC and dosages. The established procedure is demonstrated to be applicable with 80% accuracy. Results of the quality control revealed 100% conformity to all the tests for all tested drugs from all lots. The elaborated procedure is therefore applicable, rapid, effective and simple.

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**Keywords:** Anti-tuberculous, Quality control, Analytical procedure.

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## 1. Introduction

Human tuberculosis is a chronic bacterial infection, whose causative agent is *Mycobacterium tuberculosis*, (Talbert, et al., 2009). It constitutes a serious public health problem in developing countries, (OMS, 2010). According to WHO, sub-Saharan Africa and South-East harbour two third of all reported cases of tuberculosis in 2009. In 2011, 4320 cases of diverse forms of tuberculosis were screened out in Benin, (Gninafon, et al., 2011).

Fifty years ago, no medicine was available to treat tuberculosis (OMS, 2010). However, enormous progress has been observed in the development and the use of anti-tuberculous in the current world. Tuberculosis treatment follows a long duration standardized regimen of about 6 to 8 months, (Ministere De La Sante, 2008). Such a treatment requires drugs of good quality in order to increase the rates of therapeutic success and thus reduce the pandemic of this highly contagious disease.

Currently, tuberculosis treatment is based on the Associations to Fixed Doses of anti-tuberculous containing 2, 3 or 4 active principles. These presentations are made to ensure that the patient takes at once all the necessary medicines. However, cases of therapeutic failure do exist and can be attributed to several factors such as: poor functioning of the National Programs of Tuberculosis Control (PNT), the emergence of resistant strains, poor management of the stocks of medicines, absence of supply in good quality products etc. The purchasing of good quality anti-tuberculous is achieved by prequalification agencies established by WHO and adopted by the PNT (for the case of Benin). Nevertheless, drugs can undergo deteriorations for various reasons in the Centres of Screening and Treatment (CDT) where good storage practices are not always respected. Quality control is therefore necessary and should be conducted regularly in order to ensure that tablets prescribed to patients remain of good quality. In Benin, no laboratory has ever carried out a study on quality control of anti-tuberculous.

Therefore, the current study aimed to develop a method of dosage by High Performance Liquid Chromatography coupled with the dissolution of the association to fixed Doses (ADF) of 4 anti-tuberculous (Ethambutol / Rifampicin / Isoniazid / Pyrazinamide "ERHZ") used in Benin.

For each anti-tuberculous active principle, there is an individual method of identification, dosage, dissolution but rarely a single method for combinations of 2 or 3 active principles. With the advent of ADF methods with up to 4 active principles, the previous individual procedures are proven to be too slow and expensive.

## 2. Materials and methods

### 2.1. Materials

Five lots of samples were collected and identified with their batch numbers because each lot was collected from a specific batch. Each lot contained at least eighty tablets. The reference substances of the collected molecules were used. The rest of materials were essentially composed of reagents, solvents, glassware and laboratory consumables.

**Table 1**

Sample size for each lot.

Lots	Batch number	Sample size
Lot 1	GD 08002	80 tablets
Lot 2	GD 08003	160 tablets
Lot 3	GD 08141	140 tablets
Lot 4	GD 08175	300 tablets
Lot 5	ERF 110 B	1200 tablets

### 2.2. Methods

An analytical protocol of quality control of the anti-tuberculous combination ERHZ was elaborated. The protocol was applied on the lot ERF 110 B for a pilot experimentation. To each of the 9 technicians A, B, C, D, E, F, G, H, I, the elaborated protocol was sent alongside 120 tablets being 4 blisters of 30 tablets of the lot ERF, for the execution of this protocol.

The sampled medicines underwent two different laboratory tests per lot. These tests allowed to verify the quality of the drugs and to elucidate whether the active principles mentioned on the label are actually present in the medicine.

After validation of the elaborated protocol, the various samples were subjected to quality control tests. Thereafter, the four lots GD 08002, GD 08003, GD 08141 and GD 08175 collected from the CDT were tested.

Different pharmacopeia were used as well (Pharmacopée Européenne, 2002; Pharmacopée Britannique, 2002)

Overall, the analysis of the therapeutic combination Ethambutol-Rifampicin-Isoniazid-Pyrazinamide was performed following the stages described in the diagram below:

#### 2.2.1. Determination of the active principles content

This was achieved using a method of analysis that serves to determine the quantity of active principle contained in the medicine. The used method is the HPLC coupled with the UV (UV spectrophotometer) detector.

Conformity criteria:

The conformity of the two systems includes calculations of resolution and variation coefficients. The conformity of the systems is considered satisfactory when:

- Method A (Isoniazide, Pyrazinamide, Ethambutol chlorhydrate) the resolution between the peaks of Isoniazid and Pyrazinamide in the conformity solution detected at  $\lambda = 270 \text{ nm}$  is  $\geq 2$ .
- Method B (Rifampicin) the resolution between the peaks of Rifampicin and Quinone Rifampicin in the conformity solution detected at  $\lambda = 254 \text{ nm}$  is  $\geq 4$ .

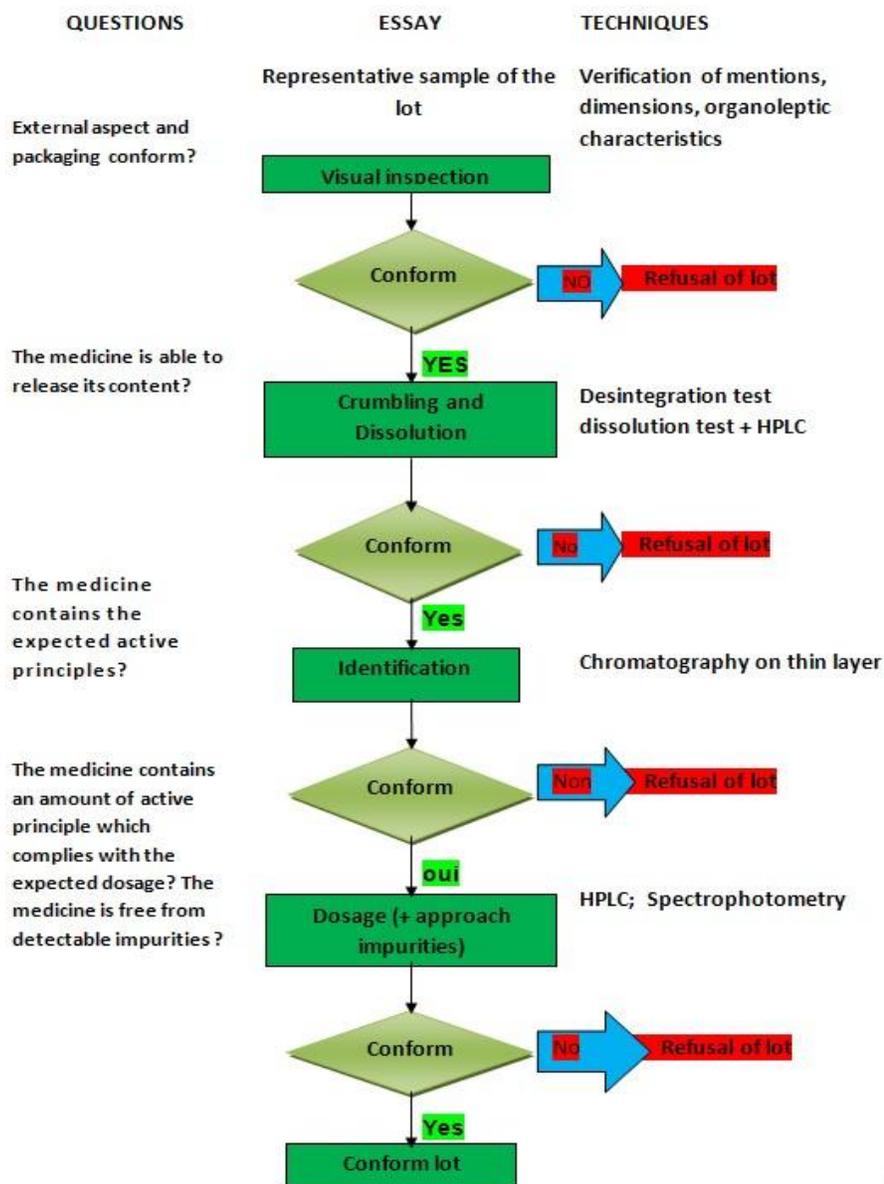


Fig. 1. Analytical procedure (9)

**Table 2**  
Chromatographic conditions.

Method	Compounds	Column	Flow (ml/min)	$\lambda$ (nm)
A	Isoniazide Pyrazinamid Ethambutol Chlorhydrate	ODS*; l = 100 mm $\Phi$ = 4.6 mm; 5 $\mu$ m	1.0	270
B	Rifampicin	ODS*; l = 250 mm $\Phi$ = 4.6 mm; 5 $\mu$ m	1.0	254

\*the use of the same column (ODS 100 mm x 4.6 mm 5  $\mu$ m or 250 mm x 4.6 mm 5  $\mu$ m) for the implementation of the two methods, A and B is possible.

### 2.2.2. Dissolution test

The percentage of dissolved active principle was determined per duration in a liquid maintained at 37 °C ± 1 °C. The steps below were followed:

- Prepare the dissolution media,
- Introduce in six hemispheric bottom cylindrical containers of 900 ml, a phosphate buffer (dissolution media) of 10 mMol at a pH of 6.8. This buffer was prepared by dissolving 8.4 g of disodic phosphate anhydride in 6 L of gas free distilled water (obtained by filtering on a filter paper of 0.45 µm with a vacuum),
- assemble the device,
- put pallets under tension and set the speed at 100 rpm,
- switch on the thermo-regulator and heat the water bath to 37 °C,
- introduce a thermometer in each of the hemispheric bottom cylindrical containers and record the temperature,
- weigh and record each of the 6 randomly chosen tablets of the lot being analysed (lot ERF 110 B) and assign a tablet to every cylindrical container,
- introduce each weighed tablet in respective measuring cylinders (1 to 6) with 2 min interval between insertion when the temperature of the media reaches 37 °C ± 0.5 °C,
- After 43 min, set the necessary device (a syringe for withdrawal) to every measuring cylinder,
- After 45 min, collect a sufficient volume of the dissolution media,
- put at the tip of each syringe a 0.45 µm Acrodisc® filter or equivalent and eliminate the first two millilitres and filter in dark brown flasks.

The filtered solution constitutes the solution to be tested.

Rifampicin being sensitive to light, its content was quickly determined by HPLC.

The selected solutions were introduced in vials. Every vial was injected twice. The solutions were eluted through the same mobile phases on the corresponding columns. The mobile phase A was used to determine Isoniazid, Pyrazinamide, and Ethambutol contents while the phase mobile B was used for Rifampicin content. The previously used method for the calculation of active principle content for the dosage remained the same. That operation was conducted for lots GD 08002, GD 08003, GD 08141 and GD 08175. The tablets of Rifampicin, Isoniazid, Pyrazinamide and Ethambutol hydrochlorate do not contain less than 90% and not more than 110% of the quantity corresponding to each of the active principles. Tolerance (Q) ≥ 80%.

Preparation of control solutions:

- control solution method A:

10 mg of Isoniazid, 40 mg of Pyrazinamide and 30 mg of Ethambutol hydrochlorate were dissolved in 10 mMol phosphate buffer at pH 6.8 and homogenized and the final reaction volume was adjusted to 100 ml with the same solvent. Thereafter, two independent solutions T1 and T2 were prepared.

- control solution method B:

20 mg of Rifampicin was dissolved in 10 mMol phosphate buffer at pH 6.8 and homogenized then completed with the same solvent to 100 ml. Two independent solutions T1, T2 were then prepared.

### 2.2.3. Numerical test for outliers (OMS Tuberculose, 2010; Pharmacopée Britannique, 2002)

Outliers are atypical observations that are distant from the data set and are isolated points or small groups of points due to errors that might occur during calculation copies and unit changes.

Two different tests were used in this study: Cochran test and Grubbs test.

- ✓ The Cochran test (C) was used to detect dispersed outliers with the formula:  $C = \frac{S^2_{max}}{\sum_{i=1}^p S_i^2}$

S<sub>max</sub> = the highest standard deviation. S<sub>i</sub> = standard deviation of a variable.

If the calculated C value is greater than the tabulated critical value C<sub>c</sub>, (C > C<sub>c</sub>), the incriminated analyst measure is rejected and the test is repeated with other standard deviations for as long as the C value is still greater than the tabulated value.

- ✓ Grubbs test (G<sub>i</sub>) is used to detect the means of outliers with the formula below

$$G_i = \max \frac{|x_i - \bar{x}|}{s_x}$$

$x_i$ : The furthest variable of the mean;  $\bar{x}$ : the mean of variables ;  $s_x$  : standard deviations

If the calculated  $G_i$  is greater than the tabulated value ( $G_i > G_c$ ), the incriminated analyst measure is rejected and the test is repeated with other means for as long as the  $G_i$  value is still greater than the tabulated value.

Excel 2010, XLSAT 2012 and GraphPad Prism were used for calculations.

### 3. Results

The methods' validation results are shown in Table III.

**Table 3**

Success rate of the analytical procedure.

<b>Cochran Test</b>	10/10	10/10	9/10	10/10
<b>Grubbs Test</b>	10/10	10/10	9/10	10/10
<b>Proportion of success per step</b>	10/10	10/10	8/10	10/10
<b>Success rate per step</b>	100 %	100 %	80%	100 %
<b>Success rate for the whole procedure</b>	80 %			

Table IV shows the results of the analyses of the five lots of the association ERHZ of anti-tuberculous used in Benin.

**Table 4**

Analyses of the lots of anti-tuberculous.

<b>Lot</b>	<b>Preliminary tests</b>	<b>Identification</b>	<b>Dosage</b>	<b>Dissolution + HPLC</b>	<b>Results</b>
<b>ERF 110B</b>	Conform	Conform	Conform	Conform	Conform
<b>GD 08002</b>	Conform	Conform	Conform	Conform	Conform
<b>GD 08003</b>	Conform	Conform	Conform	Conform	Conform
<b>GD 08141</b>	Conform	Conform	Conform	Conform	Conform
<b>GD 08175</b>	Conform	Conform	Conform	Conform	Conform
<b>Conformity Proportion</b>					100%

### 4. Discussion

The interest of this study is justified by the increased use of anti-tuberculous in the world and the necessity to develop an analytical procedure for the quality control of the association of fix doses of anti-tuberculous ERHZ in Benin. Identification by CCM reveals that the 5 samples contain all four active principles such as Ethambutol Hydrochlorate, Rifampicin, Isoniazid and Pyrazinamide. A conformity of 100% was recorded. These products contain all the four active principles which is a compulsory condition to expect a therapeutic effect in tuberculosis treatment. The absence of any of these active principles can lead to therapeutic failure, development of resistance to the treatment, an aggravation of the patient's status, or patient's death.

The dosage by HPLC coupled with the in vitro dissolution allows to quantify the amount of active principle that is freed (in vivo) over time. Various results were obtained from this study.

For the analysis of the lot ERF 110 B, the dissolution test revealed that one technician out of ten got non-compliant results (integration error for Isoniazid; technician H); two technicians out of ten didn't master the fourth part of the protocol (dissolution) of quality control of the ERHZ association. However, the three other parts were well mastered by all analysts.

The technician H is the only one to have declared a non-compliant lot. He repeated his manipulations twice before handing over the results. This situation could be explained by the fact that he encountered some difficulties during analyses: the time for taking the solutions into the measuring cylinders was lower than 45 min, use of contaminated column, problems related to peaks integration which are the most commonly encountered ones because due to the computer system.

This study revealed a conformity rate of 100% for the dissolution of each of the 4 active principles of the 5 analysed lots. These results are constant with those of Ashokraj et al. (2005). Their study aimed to verify the stability of the physical and chemical properties and the dissolution of medicines previously stocked in accelerated conditions (40 °C; 75% of relative humidity) for six months (Ashokraj et al., 2005)

The method of dissolution coupled with HPLC consists in determining Rifampicin content in the ADF, whereas the developed method allows to determine the four active principles ERHZ. The latter is faster and more efficient. In 2004, Ashokraj et al. reported 100% of conformity rate for quality control of four formulations of anti-tuberculous A, B, C and D (Ashokraj et al., 2004)

Formulation A contained one active principle (Rifampicin), the B two active principles (Rifampicin, Isoniazid), the C three active principles (Rifampicin, Isoniazid, Pyrazinamide) and the D four active principles (Rifampicin, Isoniazid, Pyrazinamide, Ethambutol Hydrochlorate). The four formulations were manufactured by four different manufacturers (Lupin Ltd, Novartis Enterprise, Pharmamed and Svizzera) and used for the dissolution in four different dissolution media (HCl 0.1 N, HCl 0.01 N, phosphate buffer and plant oil at 20%) with four different shaking rates. The formulations were subjected to accelerated stability tests for 4 weeks (40°C / 75% of relative humidity) and assessed for their chemical, physical stability and dissolution. All tested formulations passed with success the quality control tests and conform to the requirements of pharmacopeia and confirmed stable for at least 4 weeks in the packaging conditions recommended by the manufacturer.

That method presents some differences with the one used in the current study. One of these differences is the method of determination of active principles' content. In this study, ERHZ contents was determined simultaneously and R contents separately. Whereas in the other study RZE contents were determined simultaneously and H content separately.

Many other methods of dissolution were elaborated and published.

In 2003, Danckwerts et al. carried out dissolutions coupled with HPLC. The aim of their study was to optimize the conception of a formulation of a powder of ERHZ by comparing the profiles of dissolution of the formulation of tablets of ERHZ and several ERHZ formulations of instant powder (Danckwerts et al., 2003)

They reached a conclusion according to which a powdered formulation of anti-tuberculous (to be reconstituted in water before administration to patients) was optimized. The dissolution helps to assess the speed and the quantity of active principle that leaves the digestive tract to the blood stream. The particularity of this method is that the sample was plasma, whereas the one of the current study is a tablet; only one active principle was determined against 4 for the present study; the determination of active principle content is conducted in 1h30, whereas for the current study only 45min were enough. ERHZ tablets contain between 90% and 110% of every active principle. This was confirmed by the technique of dosage by HPLC.

Dosage revealed that the five lots of samples ERF 110 B, GD 08002, GD 08003, GD 08141 and GD 08175 were all conform. These results are similar to those of Enoche, 2010 from Nigeria. This author worked on the determination of Isoniazid in a pure compound and in pharmaceutical formulations. The method used was based on a combination of Isoniazid and vanillin for a better detection with the spectrophotometer at 405 nm. Isoniazid and the vanillin form in acidic environment a yellow hydrazone that has a maximal absorption of light at a wave length of 405 nm. The author demonstrated that the determination of Isoniazid content using such method was possible. At the end of the study, Enoche got 100% conformity for the four tested formulations (Enoche, 2010). That method presents some differences with the one employed in the current study with respect to reagents and the types of devices used, however the results are similar. Khuhawar et al. (2005) reported 100% of conformity as a result of quality control of three anti-tuberculous: Isoniazid BP (Isoniazid), Myrin (Isoniazide, Ethambutol, Rifampicin), Myrin P (Isoniazide, Pyrazinamide, Rifampicine) (Khuhawar et al., 2005). These results concord with the current ones. The method used in that study is a technique of dosage by HPLC that helps to measure only Isoniazid and Pyrazinamide. It is therefore different from the one developed in the current study which allows to determine four different compounds. Jiang et al. (2002) detected Ethambutol content using liquid chromatography.

That study demonstrated that Ethambutol that doesn't absorb at 270 nm can have a maximal absorption at this same wave length when it is associated with copper ions Cu (II) (Jiang et al., 2002). This property was used thereafter for the determination of Ethambutol content in other anti-tuberculous combinations by HPLC.

Khuhawa and Rind (1998) reported similar results (100% of conformity) with the current study when testing Isoniazid BP (Isoniazid), Rambuzid (Isoniazid, Ethambutol, Rifampicin), Myrene P (Isoniazid, Pyrazinamid, Rifampicin) (Khuhawa and Rind 1998)

The method used by Khuhawa and Rind is a dosage technique by HPLC that allows to determine only Isoniazid, Pyrazinamide and Rifampicin, while the current study developed a method which assessed simultaneously Isoniazid, Pyrazinamid and Ethambutol Hydrochlorate then Rifampicin content separately.

In 2006, many countries were asked by WHO to elaborate monographs of anti-tuberculous for the account of international pharmacopeia. These monographs were supposed to be capsules and tablets containing Rifampicin, Isoniazid, Pyrazinamide and Ethambutol hydrochlorate. Various procedures were established for the quality control of these four medicines. Therefore, methods such as CCM, HPLC and infrared spectrometry were developed for identification and the dosage of these active principles. The HPLC method developed in this study helps to determine the content of Rifampicin, Isoniazid, Pyrazinamide and Ethambutol (Redelinghuys., 2006) The 100% of conformity for the four stages of the quality control of ADF ERHVS show that the dispensed products respect the norms of Good Manufacturing Practices (GMP), of good reassurance of the formal circuit, good conservation, and good respect of the regulations

## 5. Conclusion

The sector of anti-tuberculous manufacturing is a well secured system of production and dispensation that provides products of good quality. This point of providing good quality products is one of the primordial objectives of WHO.

The current study elaborated an analytical procedure applied to a pilot lot ERF 110 B with 80% success. This analytical method is simple, fast, effective, efficient, precise and easily reproducible. It was validated and used for the quality control of the ADF of anti-tuberculous sampled for this study. A conformity rate of 100% was reported for each of the four studied lots at every stage of the analysis.

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