ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
21 (1999) 347–353

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Microbiological bioassay of erythromycin thiocyanate: optimisation and validation

J.A. Bernabéu, M.A. Camacho, M.E. Gil-Alegre, V. Ruz, A.I. Torres-Suárez *

Pharmacy and Pharmaceutical Technology Department, Faculty of Pharmacy, Complutense University of Madrid, 28040 Madrid, Spain

Received 3 December 1998; received in revised form 5 March 1999; accepted 15 March 1999

Abstract

The validation of an analytical method for the quantitative determination of erythromycin thiocyanate formulated in an antibiotic preparation for veterinary use was carried out. This method is based on the microbiological method described in the European Pharmacopoeia to analyze erythromycin thiocyanate as a raw material. This erythromycin thiocyanate preparation is presented as a powder for oral administration after mixing with feed. For that reason, it was planned to validate the method for the quantitative determination of erythromycin thiocyanate incorporated both in the medicated premix and the mixture with feed. The microbiological method followed a linear model and was not proportional. The number of replicates needed to obtain a valid result was less than four in all cases. The small difference in concentration, expressed in natural logarithm detected by the method, was 0.1.

Keywords: Erythromycin thiocyanate; Microbiological assay; Inhibition zones correction; Validation

1. Introduction

According to the European Union directives in referring to the Good Manufacturing Practice and Quality Control of Drugs [1–3], validation must be applied not only in manufacturing processes but also in analysis and control methods.

The validation of analytical procedures used for raw materials and finished products is gathered in the Pharmaceutical Products Multistate Register [4–6] also and more recently it has been reviewed for ICH Harmonized Tripartite Guideline [7,8].

As far as the validation characteristics are concerned, if the analytical method is described in Pharmacopoeia, it will be proceed as follows: (a) with regard to a raw materials assay, the validation will be not necessary, although it must be checked before its routine use, enclosing information about linearity, sensitivity, precision and selectivity; (b) with regard to the finished product

* Corresponding author. Tel.: +34-91-3941723; fax: +34-913941736.

E-mail address: galaaaa@eucmax.sim.ucm.es (A.I. Torres-Suárez)

0731-7085/99/$ - see front matter
PII: S0731-7085(99)00143-0

assay, it is necessary to carry out a brief validation to demonstrate that the precision, selectivity and accuracy are appropriate.

The aim of this work was to carry out the validation of an analytical method for the quantitative determination of erythromycin thiocyanate formulated in an antibiotic preparation for veterinary use. This method is based on the microbiological method described in the European Pharmacopoeia [9] to analyze erythromycin as a raw material. This erythromycin thiocyanate preparation is presented as a powder for oral administration after mixing with feed. For that reason, the validity of the method was planned for the quantitative determination of erythromycin thiocyanate incorporated both in the medicated premix and the mixture with feed. In both cases, linearity, precision, accuracy and sensitivity of the microbiological method were studied.

Table 1
Modifications of the microbiological method and slope values of the calibration curve obtained in each case

pH	Disc size (mm)	Range of concentrations (μg ml^{-1})	Slope (mm)
6.4	6	0.64–1.56	Inhibition zone was not observed
6.4	6	0.5–4.5	Inhibition zone was not observed
6.4	6	5–25	2.1
6.4	6	5–45	2.5
6.4	12.7	5–45	2.3
8.0	6	5–45	Inhibition zones were overlapped
8.0	6	0.5–4.5	3.5
8.0	12.7	0.5–4.5	3.0
8.5	6	0.5–4.5	3.9
9.0	6	0.3–2.5	4.7

Table 2
Inhibition zone diameter and corrected diameter obtained in the calibration curve of the erythromycin thiocyanate

Dish	Calib. conc. (μg ml^{-1})[a]	Inhibition zone diameter (mm)				
		X_1	X_2	X_3	M[b]	H_i[c]
1	0.3	14.0	13.5	14.5	17.5	13.8
2	0.3	13.0	14.0	14.0	16.5	14.3
3	0.3	14.0	13.5	14.5	17.5	13.8
4	0.7	16.0	16.5	16.5	17.5	16.1
5	0.7	16.0	15.5	15.5	17.0	15.9
6	0.7	16.0	15.5	16.0	17.0	16.0
7	1.5	19.5	19.5	20.0	17.5	19.3
8	1.5	18.5	19.0	19.5	17.0	19.2
9	1.5	19.5	19.5	20.0	17.5	19.3
10	2.5	21.5	21.5	20.5	17.0	21.4
11	2.5	21.5	21.5	21.0	17.5	21.0
12	2.5	21.0	20.5	21.5	17.0	21.3

[a] Calib. conc., concentration of the calibration curve.
[b] M, reference concentration.
[c] H_i, corrected diameter.

Table 3
Premix: Linearity, regression line parameters and regression variance analysis

Total number of samples	12
Range of linearity (μg ml^{-1})	0.3–2.5
Correlation coefficient '*r*'	0.9701
Determination coefficient 'r^2'	0.9468
Slope '*b*'(mm)	3.5
Standard deviation of '*b*' (mm)	0.2
Intercept '*a*' (mm)	17.5
Standard deviation of '*a*' (mm)	0.1
C.V. residual (%)	2.80
Residual sum of squares	5.26
ANOVA test '*F*' regression	391.40*
'*F*' linear model	3.50
'*t*' to test of proportionality	175.27*

* $P<0.01$.

Table 4
Sensitivity parameters

Calibration sensitivity (mm)	3.5
Mean analytical sensitivity	21.5
Discriminatory capacity	0.1

Table 5
Diameter of the inhibition zones obtained in the calibration curve of erythromycin thiocyanate in a mixture with feed and corrected inhibition zones of the calibration curve

Dish	Calib. conc. (μg ml^{-1})	Inhibition zones diameter (mm)		
		X_1	M	H_i
1	0.3	13.5	17.5	13.8
2	0.3	12.5	17.0	13.1
3	0.7	16.0	17.5	16.3
4	0.7	17.5	18.0	16.8
5	1.5	19.0	17.5	19.3
6	1.5	19.0	16.5	20.5
7	2.5	20.0	16.5	21.6
8	2.5	20.5	17.0	21.5

2. Materials and methods

2.1. Materials

The raw materials were erythromycin thiocyanate, animal feed and dextrose, and were supplied by Andrés Pintaluba Veterinary Products.

The medicated premix is composed of erythromycin thiocyanate and dextrose (20:80). The reconstituted mixture consists of premix and feed; 25 kg of the feed includes 750 g of premix. The ratio erythromycin thiocyanate-feed is 1:166.

The test-agar was no. 1 Difco laboratories and the microorganism was *Bacillus subtilis* ATCC 6633.

2.2. Methods

2.2.1. Optimisation of the microbiological method

The quantitative measurement of the erythromycin thiocyanate was carried out through diffusion in a test-agar using *Bacillus subtilis* ATCC 6633 as an indicator microorganism. The test-agar was the one described in the European Pharmacopoeia as A-Agar for the biological assay of antibiotics. The freeze-dried microorganism was reconstituted with sterile physiological saline. From this suspension, the agar slants were prepared, and from these ones, a Roux bottle. The spores concentration in the obtained suspension was fitted to a 25% of transmittance at 580 nm. For the biological assay of erythromycin thiocyanate, 0.15 ml of this suspension was inoculated in 15 ml of test-agar for each dish.

The standard curve was determined using five different concentrations of erythromycin thiocyanate, obtained from dilutions of an initial 1 mg ml^{-1} erythromycin thiocyanate solution in methanol.

Table 6
Mixture: Linearity, regression line parameters and regression variance analysis

Total number of samples	8
Linear range (μg ml^{-1})	0.3–2.5
Correlation coefficient '*r*'	0.9922
Determination coefficient 'r^2'	0.9844
Slope '*b*'(mm)	3.9
Standard deviation of '*b*' (mm)	0.2
Intercept '*a*' (mm)	18.1
Standard deviation of '*a*' (mm)	0.2
C.V. residual (%)	2.55
Residual sum of squares	1.24
ANOVA test '*F*' regression	378.08*
'*F*' linear model	0.53
'*t*' to test proportionality	112.13*

* $P<0.01$

Table 7
Erythromycin thiocyanate content, expressed as concentration and percentage respect to the theoretical value, in a sample of the premix and the mixture assayed the same day

Theoretical conc. (μg ml^{-1})	Premix		Mixture	
	Exp. conc.[a] (μg ml^{-1})	%	Exp. conc. (μg ml^{-1})	%
0.7	0.67	95.71	0.65	92.86
	0.70	100.00	0.67	95.71
	0.65	92.86	0.74	105.71
	0.65	92.86	0.69	98.57
1.5	1.47	98.00	1.46	97.33
	1.53	102.00	1.50	100.00
	1.48	98.67	1.51	100.67
	1.48	98.67	1.51	100.67

[a] Exp. conc., experimental concentration.

In order to assay these standard samples, 12 dishes were used, three for each concentration, with the exception of one which was taken as a reference and set in every dish. Four paper discs were placed in each dish and an exact measured volume of the corresponding dilution was deposited: 20 μl for paper discs of 6 mm diameter, and 100 μl for paper discs of 12.7 mm diameter. In this way, each dish contained three paper discs with the corresponding antibiotic concentration and one paper disc with the reference concentration.

The dishes were incubated, for 17 ± 1 h at 33.5 ± 1.5°C and the diameter of the inhibition zone of the microorganism growth was measured.

2.2.1.1. Correction of the inhibition zone with respect to the reference concentration. To fit the obtained data, the following equation was applied:

$$H_i = X_i \cdot M / M_i$$

where: M, average value of the reference inhibition zones from the 12 dishes; M_i, reference inhibition zone in the studied dish (i); X_i, average value of the inhibition zone of the standard sample in the studied dish (i); and H_i, corrected inhibition zone value of the standard sample in the studied dish (i).

Therefore, three corrected inhibition zones values were obtained for each concentration except for the reference one since it was used to carry out the correction.

The correlation between the variables, corrected inhibition zone value (H_i) and concentration (C) is semilogarithmic.

$$H_i = m \ln C + a$$

where m is the slope and a is the intercept.

In order to increase the sensitivity method, different changes were made with respect to the following factors: (a) paper disc size; (b) range of concentration; and (c) test agar pH.

Table 8
Percentage with respect to the theoretical value of erythromycin thiocyanate content in a sample of premix and mixture which was assayed four different days

	Erythromycin thiocyanate (%)	
	Premix	Mixture
Day 1	95.71	92.72
	98.00	96.07
Day 2	84.28	98.33
	102.01	92.00
Day 3	108.60	96.47
	98.67	97.60
Day 4	100.56	95.21
	103.17	96.92

Table 9
Statistical parameters of the precision and accuracy study

	Premix		Mixture	
Theoretical concentration (μg ml^{-1})	0.7	1.5	0.7	1.5
Recovery (%)	95.36	99.33	–	–
C.V. repeatability (%)	3.54	2.56	1.81	1.59
No. determinations*	3.31	1.74	0.87	0.67
Snedecor *F*-test	4.32	4.32	–	–
Mean recovery (%)	97.35	97.35	–	–
Student's *t*-test	2.28	2.28	–	–
C.V. intermediate precision (%)	7.15	7.15	2.37	2.37

* $P < 0.05$.

2.3. Validation [10]

The linearity study was made on two calibrated curves prepared from:

1. Erythromycin thiocyianate standard (curve of calibration for the analysis of the premix samples),
2. Erythromycin thiocyanate standard mixed with feed (curve of calibration for the analysis of the mixture). In order to avoid the possible influence of feed constituents on the microorganism growth, the calibration curve used to assay the samples of the mixture was prepared from a mixture of erythromycin thiocyanate and feed.

Linearity of the model was verified by analysis of variance (Anova), and the proportionality of the method by Student's *t*-test with respect to the theoretical value zero. The value of the residual coefficient of variation (CV) quantifies the goodness of the fitting of the experimental data to a straight line.

The following parameters were determined for studying the sensitivity of the microbiological method: the calibration sensitivity which corresponds to the slope (as it is a linear model); the mean analytical sensitivity, considered as a quotient between the calibration sensitivity and mean standard deviation for all concentrations; and the discriminatory capacity which corresponds to the inverse of the analytical sensitivity multiplied by Student's *t* value with a probability of 0.05 and degrees of freedom corresponding to the number of the samples.

The study of repeatability was carried out for both premix and mixture samples analysed on the same day at two different concentrations (0.7 and 1.5 μg ml^{-1}).

In the intermediate precision study, a sample of premix and mixture was analysed twice in 4 days at two different concentrations (0.7 and 1.5 μg m^{-1}).

The accuracy study was only carried out for samples of the premix since the mixture was assayed from its calibration curve. The average percentage of recovery of the analyte was calculated. The method was considered accurate, when through an Student's *t*-test, the mean value of recovery was not different from 100%.

2.3.1. Preparation of samples for linearity and sensitivity evaluation

2.3.1.1. Calibration curve to assay the samples of the premix. Erythromycin thiocyanate (100 mg) was dissolved in 100 ml methanol in a volumetric flask. The concentration of the resulting solution was 1 mg ml^{-1}. This solution was diluted to 1:100 proportion with phosphate buffer (composed of potassium dihydrogen phosphate and dipotassium hydrogen phosphate trihydrate, pH 7.9; 0.1 M). Different amounts of this solution was transferred to 10 ml volumetric flask and was diluted with the same buffer to obtain desired concentration (0.3–2.5 μg ml^{-1}).

2.3.1.2. Calibration curve to assay the samples of the premix. Approximately 17.06 g of animal feed was mixed with 100 mg of erythromycin thio-

cyanate and then 100 ml of methanol were added. The solution was stirred for 15 min and then centrifuged at 3750 rpm for 10 min. As the concentration of erythromycin thiocyanate in the supernatant was 1 mg ml^{-1}, the range of the desired concentrations (0.3–2.5 μg ml^{-1}) was obtained by dilution with phosphate buffer.

2.3.2. Preparation of samples for accuracy and precision study

2.3.2.1. Premix. Like the reference sample, 500 mg of the premix (which corresponds to 100 mg of erythromycin thiocyanate) were dissolved in 100 ml of methanol followed by a 1:100 dilution with phosphate buffer. Different amounts of solution was transferred to 10 ml flask and diluted with phosphate buffer to obtain desired concentration (0.7–1.5 μg ml^{-1}).

2.3.2.2. Mixture. A total of 600 g of the mixture were mixed in a V blender for 15 min and then pulverized in a mill with a view to improve the extraction of the antibiotic. To 17.6 g of this powdered produce, 100 ml of methanol was added following the same method as it was in the case of the calibration curve for the assay of the mixture, but only two concentrations were prepared (0.7–1.5 μg ml^{-1}).

2.3.3. Assay of the sample for the precision and accuracy study

To assay the samples, eight Petri dishes (two for each concentration of the calibration curve) were used, and four discs per Petri dish (a–d): (a) solution with reference concentration (1 μg ml^{-1}); (b) solution with a concentration of the calibration curve (0.3; 0.7; 1.5; 2.5 μg ml^{-1}); (c) Sample no. 1; and (d) Sample no. 2.

The concentration of the samples would be:

- 0.7 μg ml^{-1} in those plates where the concentration of the calibration curve was 0.3 and 0.7 μg ml^{-1}.
- 1.5 μg ml^{-1} in those plates where the concentration of the calibration curve was 1.5 and 2.5 μg ml^{-1}.

3. Results and discussion

3.1. Optimisation of the microbiological method

Table 1 shows the method changes and the slope values of the calibration curve obtained in each case. The slope value is the measurement of sensitivity. As it can be observed, modifying both the paper disc diameter and the concentration of erythromycin thiocyanate does not lead to a significant improvement in sensitivity, whereas the pH modification of the test-agar is shown as the most important factor to increase the slope, and because of this, the sensitivity of the microbiological method.

Therefore the analytical conditions of the method were:

- Microorganism: *Bacillus subtilis* ATCC 6633
- Test-agar: A (European Pharmacopoeia)
- Test-agar pH: 9
- Diameter of the paper discs: 6 mm
- Range of erythromycin thiocyanate concentration: 0.3; 0.7; 1.0 (Reference concentration); 1.5 and 2.5 μg ml^{-1}
- Incubation temperature of the dishes: 33.5 ± 1.5°C.
- Phosphate buffer: composed of potassium dihydrogen phosphate and dipotassium hydrogen phosphate trihydrate (pH 7.9; 0.1 M)

3.2. Linearity and sensitivity

Table 2 represents the values of the inhibition zones from the calibration curve prepared with reference erythromycin thiocyanate and the inhibition zones of the growth of microorganism of this calibration curve after correction. From these values, the parameters of the regression line are calculated and shown in Table 3.

As observed, the correlation between the two variables, diameter of the corrected inhibition zone and natural logarithm of the concentration, gives a correlation coefficient value of 0.9701, which indicates that 94.68% of the variation in the y axis is explained by variation in the x axis.

On the other hand, it is observed that the correlation between these two variables fits to a linear model from the low value of residual CV and the F-value. The method was not propor-

tional due to a significant difference between the intercept and the zero value observed through the Student's t-test.

The results obtained from the calculation of the sensitivity parameters of the microbiological method are displayed in Table 4. The small difference of concentration detected by this method and expressed in a natural logarithm equals 0.1.

The diameter of the inhibition zones from the calibration curve of erythromycin thiocyanate in the mixture and the corrected values is shown in Table 5.

Table 6 shows the parameters of linear regression and analysis of variance of the regression for the mixture.

The correlation between the two variables, diameter of the corrected inhibition zone and natural logarithm of the concentration is shown through a correlation coefficient value of 0.9922.

On the other hand, it is observed that the correlation between these two variables follows a linear model from the F value. The residual CV was 2.55%. The method was not proportional as shown by the t-test.

3.3. Precision and accuracy

Table 7 shows the data obtained from the repeatability study for the premix and mixture.

From the values of the premix expressed as percentages of the theoretical concentration (Table 7), the accuracy was studied.

Table 8 shows the results obtained from the intermediate precision study and the Table 9 represents the statistical parameters of the study of precision and accuracy of the microbiological method.

It is observed that the number of replicates needed to obtain a valid result with an acceptable level of 0.05 is lower than 4 in all cases. This indicates that four replicates were sufficient to obtain a reliable value using this analytical method.

Using the Snedecor F-test, it is verified that the concentration of the analyte does not affect the variance of the results. For this reason, the mean percentage recovery for both studied concentrations was calculated and it was found to be 97.35% as shown in Table 9.

The value of the Student's t-test obtained demonstrates that the method was accurate when erythromycin thiocyanate was assayed in premix since no significant differences between 100% and mean recovery value were detected.

4. Conclusions

From the results, it can be concluded that the proposed microbiological method was valid and suitable for the quantitative determination of erythromycin thiocyanate in medicated premix and in mixture with feed.

Acknowledgements

This work has been supported by Andrés Pintaluba Veterinary Products.

References

[1] European Union Regulations for the good manufacturing and quality control of medicinal products 75/319.
[2] European Union Regulations for the good manufacturing and quality control of medicinal products 89/381.
[3] European Union Regulations for the good manufacturing and quality control of medicinal products 91/356.
[4] European Union Multi-state register of pharmaceutical products 75/318.
[5] European Union Multi-state register of pharmaceutical products 89/341.
[6] European Union Multi-state register of pharmaceutical products 91/507.
[7] ICH Harmonised Tripartite Guideline CPMP/ICH/281/95.
[8] ICH Harmonised Tripartite Guideline CPMP/ICH/381/95.
[9] Microbiological assay of antibiotics. European Pharmacopoeia, 3rd ed., Council of Europe, Maisonneuve, Sainte Ruffine.
[10] M.A. Camacho, A.I. Torres, M.E. Gil, M.M. Obregón, V. Ruz, STP-Pharma 3 (1993) 197–202.