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Simultaneous determination and validation of antimicrobials in plasma and tissue of actinomycetoma by high-performance liquid chromatography with diode array and fluorescence detection

Norma Cavazos-Rocha [a], Lucio Vera-Cabrera [b], Oliverio Welsh-Lozano [b], Noemí Waksman-de-Torres [a], María de la Luz Salazar-Cavazos [a,*]

[a] *Department of Analytical Chemistry, Facultad de Medicina, UANL, Mexico*
[b] *Department of Dermatology, Universitary Hospital, Madero y Aguirre Pequeño Col. Mitras Centro, C.P. 64460 Mty, N.L., Mexico*

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Abstract

A simple, precise, and reliable chromatographic method was developed for the simultaneous determination in plasma and infected tissue of five antimicrobials proposed for the treatment of actinomycotic mycetoma: amoxicillin, trimethoprim, linezolid, sulfamethoxazole and garenoxacin. Separation of the analytes was achieved on an Atlantis dC18 column (150 mm × 4.6 mm, ID 5 μm) with a mobile phase composed of acetonitrile and trifluoroacetic acid (ATF) 0.1% (v/v) using a gradient program. The detection was carried out using a diode array detector at 254 nm and in a fluorescence detector at wavelengths of excitation and emission of 292 nm and 392 nm for linezolid and sulfamethoxazole, and 292 nm and 408 nm for garenoxacin, respectively. The intraday precision was in the range of 0.7–15% of relative standard deviations (%R.S.D.) for plasma and 1–18% for tissue. Linearity range was from 2.4 to 20 μg/ml for amoxicillin, 0.3 to 20 μg/ml for trimethoprim, sulfamethoxazole and linezolid, and 0.3 to 10 μg/ml for garenoxacin. Acetonitrile was used to precipitate proteins from plasma. Recoveries in plasma ranged from 71% to 118% and in infected tissue from 78% to 122%. Limits of detection (LODs) were 1.2 and 0.5 μg/ml for amoxicillin in plasma and tissue, respectively and 0.15 and 1.2 μg/ml in plasma and tissue, respectively for the other antimicrobials. The method can be applied for individual or simultaneous determination of the antimicrobials in plasma and tissue of mouse infected with actinomycetoma.

Keywords: Amoxicillin; Trimethoprim; Garenoxacin; Sulfamethoxazole; Linezolid; Actinomycetoma; Antimicrobials by HPLC

1. Introduction

Mycetoma is a chronic infectious disease that affects skin, subcutaneous cellular tissues, bone, and adjacent organs. It can be produced by fungi (eumycetes) or bacteria. In Mexico, most cases (98%) are produced by aerobic actinomycetes and about 86% are produced by *Nocardia brasiliensis* [1].

Therapy for actinomycetoma has been based on the use of sulfonamides, particularly sulfamethoxazole in combination with trimethoprim (SXT) [2]. During the past 15 years, we have used the combination of SXT with amikacin in severe cases of mycetoma or those affecting important organs, such as those in the back, which can spread and affect the lungs or compress the spinal cord. The cure rate obtained with this combination is around 95% [3].

To have therapeutic alternatives, other antimicrobials have been tested *in vitro*. In these assays, it has been observed that *N. brasiliensis* isolates are susceptible to aminoglycosides, including amikacin, gentamycin, isepamycin; quinolones such as gatifloxacin, moxifloxacin, and ciprofloxacin; Beta-lactam compounds like amoxicillin-clavulanic acid, piperacillin-tazobactam, ticarcillin-clavulanic acid; and oxazolidinones such as linezolid, DA-7157, and DA-7867 [4–6]. Some of these antimicrobials have been tested *in vivo* in an experimental mouse model of actinomycetoma, and showed good activity, particularly in the case of linezolid [4]. However, the doses for the experiments *in vivo* were selected based on previous studies with other microorganisms; to better interpret the *in vivo* results it is necessary to determine the pharmacokinetics of the drugs. This would allow us to know the plasma levels

* Corresponding author. Tel.: +52 81 83294185; fax: +52 81 86758546.
E-mail address: masalaz@ccr.dsi.uanl.mx (M. de la Luz Salazar-Cavazos).

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reached at different doses of the agents tested, to determine the break points used in the *in vitro* assays, and to select the optimal doses to be used in the animal models of infection.

Several methods have been reported for determining levels of trimethoprim-sulfamethoxazole in serum and pharmaceutical preparations [7,8], trimethoprim in tissues [9], amoxicillin-clavulanic acid in serum, urine and tissues [10–18], linezolid in serum, biological tissues and other fluids [19–23], and garenoxacin in serum and plasma of experimental animals [24,25]. Most of these determinations were made using high-performance liquid chromatography (HPLC) [8–25]. Sulfamethoxazole and trimethoprim concentrations were determined simultaneously in pharmaceutical formulations by UV-visible spectrophotometry using derivatization [7], and amoxicillin concentration has been determined in plasma by liquid chromatography with detection by fluorescence [18]. Although methods for determining the concentrations of these agents individually have been described, a method for the simultaneous determination of these antimicrobials does not exist. In this study, we developed a chromatographic method for the simultaneous determination in plasma and tissue of amoxicillin, trimethoprim, sulfamethoxazole, linezolid and garenoxacin concentrations, in a mouse infected with *N. brasiliensis*.

2. Experimental

2.1. Reagents

Amoxicillin (AM), sulfamethoxazole (SMX), trimethoprim (TM) and trifluoroacetic acid (TFA) were obtained from Sigma–Aldrich Products (St. Louis, MO, USA), garenoxacin (GR) from Bristol Myer Squibb (Princeton, NY, USA) and linezolid (LZ) from Pharmacia and Upjohn (Kalmazoo, MI, USA). K_2HPO_4 and H_3PO_4 were from Fermont (Monterrey, México), tetrabutyl ammonium phosphate (TBA) was from Baker Analyzed (USA). All standards and chemicals were analytical-grade reagents. HPLC grade acetonitrile was purchased from Fisher Scientific (USA). Deionized water was purified by a MilliQ System (Millipore Co., USA). Before use, the mobile phase and the buffer used for the preparation of the samples were filtered through a 0.45 μm Nylon filter (Waters Corporation, Milford, MA, USA). Samples were filtered through Nylon acrodiscs (Waters).

Phosphate buffer (0.1 M; pH 7.2) was prepared as follow: 1.7418 g of K_2HPO_4 was dissolved in 800 ml of water, pH was adjusted to 7.2 with H_3PO_4 28% and made up to 1000 ml.

2.2. Equipment and chromatographic conditions

The chromatographic separation was achieved using a Waters 2690 Alliance liquid chromatograph with diode array detector 996 (DAD) and fluorescence detector 474. An Atlantis dC18 column 150 mm × 4.6 mm ID, with 5 μm particle size (Waters) was used. Column temperature was maintained at 30 °C. Samples were eluted with a mobile phase consisting of 0.1% trichloroacetic acid (solvent A) and acetonitrile (solvent B); the gradient used is shown in Table 1. The flow rate of the mobile phase was 1.0 ml/min. The injection volume was 10 μl. The DAD wavelength was set at 254 nm for all analytes. SMX, LZ and GR were also analyzed by fluorescence; for SMX and LZ the fluorescence intensities were measured at an excitation wavelength of 282 nm and an emission wavelength of 392 nm and for GR the excitation wavelength was 292 nm and an emission wavelength of 408 nm. Total running time was 30 min.

Table 1
Gradient used for elution of the antimicrobials

Time (min)	TFA 0.1%	Acetonitrile
1	92	8
4	92	8
14	73	27
24	73	27
26	92	8
30	92	8

2.3. Standard solutions

Ten milliliters of the standard stock solutions of each antibiotic were prepared in methanol at a concentration of 1 mg/ml and stored at −20 °C. From these solutions, standard stock mixtures from each antibiotic at concentrations ranging from 10 to 100 μg/ml were prepared and stored at −20 °C for 30 days; from these dilutions, the working aqueous solutions (working standard solutions) of the drugs were prepared weekly in 0.01 M phosphate buffer pH 7.2, to yield final concentrations of 0.075, 0.15, 0.3, 0.6, 2.4, 5, 10 and 20 μg/ml.

2.4. Sample treatment

Drug-free plasma pools and infected tissue with *N. brasiliensis* from Balb/C mice samples were frozen at −20 °C. Before the treatment, frozen samples were thawed to room temperature (approximately 22 °C).

2.4.1. Plasma treatment

To 50 μl of the thawed samples, different volumes from the two standard stock mixtures were added to obtain final concentrations of 0.6, 5 and 10 μg/ml. Phosphate buffer was added to achieve 200 μl. To these solutions, 200 μl acetonitrile was added to precipitate proteins, the mixture was vortexed for 5 s and centrifuged for 5 min at 5304 × *g*, and the supernatant was filtered through 0.45 μm Nylon filters (Waters). Filtrates were received into 150 μl inserts.

2.4.2. Tissue treatment

The mycetoma tissue samples obtained from different mice were mixed, homogenized and stored at −20 °C until use. Between 0.1 and 0.3 g of thawed tissue was placed in 15 ml centrifuge tubes. Different volumes from the standard stock mixture were added to obtain final concentrations of 0.4, 2 and 8 μg/ml. The samples were allowed to sit for 45 min; then, 2 ml phosphate buffer was added and vortexed for 2 min, and then 3 ml of acetonitrile was added and centrifuged for 10 min at

4000 × *g*. The supernatant was then transferred to a clean tube and a new 500 μl volume of acetonitrile was added to this supernatant, vortexed again for 10 s and recentrifuged to assure protein precipitation.

2.5. Method validation

2.5.1. Linearity

Calibration curves were constructed at six concentration levels: 0.075, 0.15, 0.3, 0.6, 2.4, 5, 10 and 20 μg/ml. The area values were plotted against the concentration and calibration plots were constructed using an external standard method. Curves were constructed from triplicate runs. According to the FDA, quantification limits were chosen from the lowest concentrations of each analyte on the calibration graphs that afforded R.S.D.s ≤20% [26].

To take into consideration the matrix effect, calibration graphs at the same concentration levels were constructed in plasma and infected tissue, both free of antimicrobials.

2.5.2. Precision

The precision of the system and method was evaluated by means of %R.S.D. of t_R and peak areas. R.S.D.s of the system were calculated by means of injection of three working mixtures at 0.6, 5.0 and 10 μg/ml, each in triplicate. Intraday precision of the method in plasma and tissue were determined by assaying three spiked samples at 0.6, 5.0 and 10 μg/ml for plasma and 0.4, 2.0 and 8.0 μg/ml for tissue. All samples were processed in triplicate. Interday precision was calculated by injecting aliquots of the same solution on different days. These samples were also injected 8 h and 24 h after their preparation to test their stability.

2.5.3. Recovery

Recoveries were evaluated by adding to 50 μl of the plasma pool the adequate volume from the stock solution of the antibiotic mixture to achieve 0.6, 5 and 10 μg/ml. The procedure for the sample preparation was the same as that previously described. Results were calculated from the calibration curves by means of the external standard method. The procedure was repeated three times. The same procedure was applied to the tissue samples with drug concentrations of 0.4, 2 and 8 μg/ml

2.5.4. Robustness

To evaluate the robustness of the method, a Placket and Burman experimental design for seven variables was used [27]. The experimental design used is shown in Table 2. The following variables were taken into consideration: the volume of injection, the temperature, the proportion of the solvents use to prepare the samples, %TFA in the mobile phase and the flow rate. In the case of the drugs that were analyzed by fluorescence, modifications in the emission and excitation wavelengths were also made. In all cases, the parameter for the evaluation was the t_R of the analyte.

Results were calculated using the following equation:

$$\text{If } |Vt_R - vt_R| > s(2)^{1/2},$$

the difference was assumed to be significant where Vt_R and vt_R are the mean of the retention times obtained from the high and low values defined for each variable respectively, and s is the standard deviation of the t_R obtained from the precision analysis. This analysis was done for each of the drugs under evaluation.

2.5.5. Selectivity

Selectivity was tested by examining seven different samples of unspiked plasma or tissue free of antibiotics to confirm that the signals originating from the matrix did not interfere with the signals from the analytes. Identification of each compound in the spiked samples was made by the retention times as well as by comparison of the DAD spectra from the chromatographic signals with those of the respective standards.

2.5.6. Stability

To determine the stability of the analytes in the matrix, the plasma pool was spiked with the standard mixture to achieve a concentration of 5.0 μg/ml for each antimicrobial; 50 μl fractions were stored at −20 °C for 6 months. Each week a sample was thawed to record a measurement. Results were evaluated using control graphs constructed with the results obtained from the precision experiment. Deproteinized plasma samples were also stored at 4 °C. The stability of each sample was tested 10 and 24 h after their preparation.

Table 2
Placket and Burman experimental design

Variable	Experiment								
	0[a]	1	2	3	4	5	6	7	8
Temperature (°C) (A)	30	32	32	32	32	28	28	28	32
Flow rate (ml/min) (B)	1.0	1.2	1.2	0.8	0.8	1.2	1.2	0.8	1.2
ATF (%) (C)	0.1	1.5	0.5	1.5	0.5	1.5	0.5	1.5	1.5
Acetonitrile (%) (D)	8	10	10	6	6	6	6	10	10
λex/λem (nm) (E)	292/392	294/394	290/390	294/394	290/390	290/390	294/394	290/390	294/394
	292/308	294/310	290/306	294/310	290/306	290/306	294/310	290/306	294/310
Vol. injection (μl) (F)	10	12	8	8	12	12	8	8	12
Solvents (G)	Buffer	ATF	ACN	ATF	ACN	ACN	ATF	ACN	ATF
Results (t_R)		S	t	U	v	w	x	y	z

[a] 0 correspond to the optimized method conditions.

3. Results and discussion

3.1. Chromatographic separation

To select the optimal chromatographic conditions, different stationary phases and elution systems were tested. In most of the proven chromatographic conditions, SMX, TM and LZ showed symmetric and reproducible signals with retention times higher than both the dead time and the signals originating from the matrices (plasma and tissue). However, AM and GR presented several problems, given their chemical characteristics, ionic pair reagents are recommended for their separation [12,14,15]. Therefore, tetrabutyl ammonium phosphate was used in the elution solvent; this reagent has been recommended both for AM [12,14,15] and several quinolones, including garenoxacin [25–27]. The polymeric X-Terra column has been used for ion-pair chromatography. Its principal advantage is a wide pH range (between pH 2 and 12) and its polar characteristics produced an improved form of the chromatographic signals. It was tested with a gradient consisting of A: phosphate buffer pH 7.2 containing TBA (0.01 or 0.015 M) and B: ACN. However, AM showed low t_R (less than 2 min) in all the conditions tested, such as changes in the gradient programs and in TBA concentration. In addition, the use of the ionic pair reagent required longer conditioning times for the column; another disadvantage is the short life of the column; for these reasons, this system was discarded. Finally, the use of the endcapped Atlantis dC_{18} column, which exhibits better retention of polar compounds with good peak shapes, permitted the separation of all the antimicrobials without using the ionic pair reagent. This was considered an advantage, as less time was required, the column life could be enhanced, and the preparation of mobile phase was more rapid.

Representative chromatograms of the standard mixture at 5 μg/ml are shown in Fig. 1 (DAD, wavelength 254 nm and fluorescence).

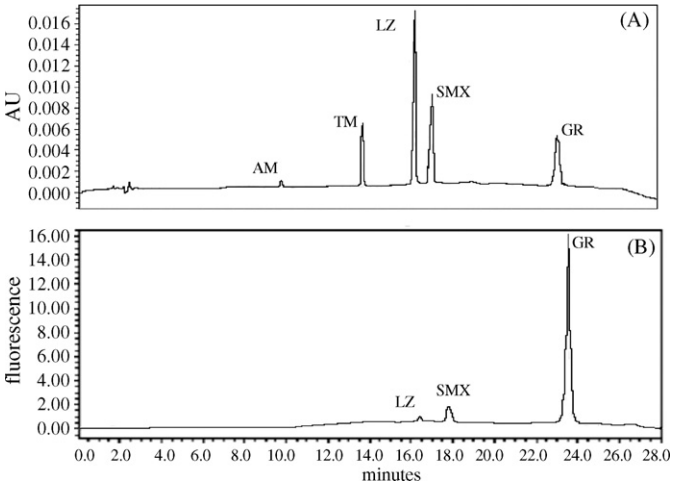

Fig. 1. HPLC chromatograms of the standard mixture: (A) detection at 254 nm. Elution order is: amoxicillin, trimethoprim, linezolid, sulfamethoxazole, garenoxacin. (B) Fluorescence detection: linezolid, sulfamethoxazole and garenoxacin. Separation conditions are described in the text.

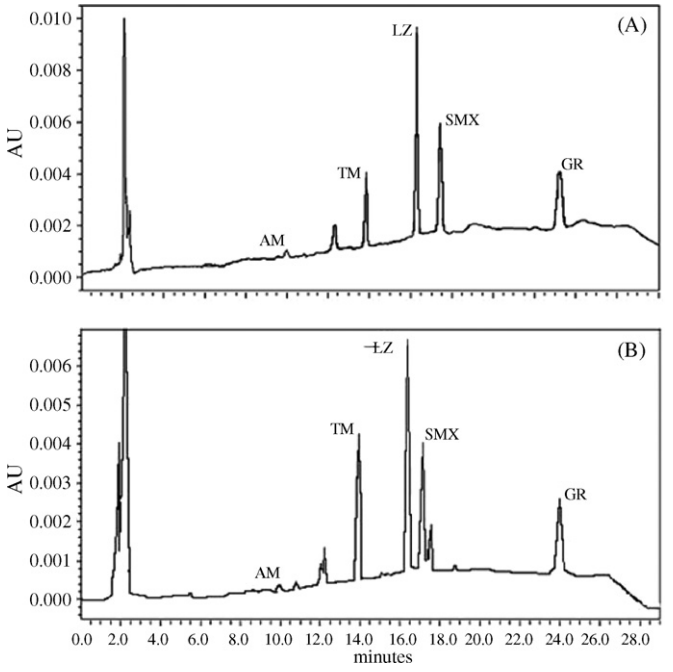

Fig. 2. HPLC chromatograms of (A) plasma and (B) infected tissue spiked with the standard mixture at 0.6 μg/ml. Elution order is: amoxicillin, trimethoprim, linezolid, sulfamethoxazole, garenoxacin. Separation conditions are described in the text.

Different agents such as ACN, perchloric acid and methanol, have been reported for protein precipitation from plasma and serum samples [10,13,14,20,22,24,25]. We evaluated the use of ACN, MeOH and TFA 0.1% for protein precipitation. The best recoveries were obtained using ACN. To optimize the conditions, 50 and 100 μl from the samples were tested. No significant differences in precision and accuracy were found, so 50 μl was used for the method validation.

For treatment of mycetoma tissues, a modification of the method reported by Brandsteterova and Luo for muscular tissue was used [9,18]. No differences were found between the extractions at two different pHs (7.2 and 7.4). For the deproteinization, a 1.5:1 ratio of ACN and buffer was used. Considering that an infected tissue contains more proteins than normal tissue, the precipitation procedure had to be repeated with the same volumes used in both phases for this precipitation step.

Fig. 2 shows the chromatographs of plasma and tissue spiked with the standard mixture at 0.6 μg/ml. The identity of the antimicrobials in the chromatogram were confirmed by means of the DAD spectra of the corresponding signals.

3.2. Method validation

Tables 3 and 4 show the results for the validation of the method in plasma and tissue.

3.2.1. Linearity

A linear response implies R.S.D.s equal to or less than 15% in the response factors. The response factors were calculated

Table 3
Validation parameters of the method used for the analysis of the antimicrobials in plasma and tissue

Antimicrobials	Linear range (μg/ml)	Intraday precision %R.S.D.			Linear equation $y = mx + b$	LOD (μg/ml)	LOQ (μg/ml)
		0.6 μg/ml	5 μg/ml	10 μg/ml			
Amoxicillin							
Plasma	2.4–20	10.0	3.1	6.5	$y = 865.6x - 365$	1.20	2.40
Tissue	1.6–20	15.0	2.0	3.0	$y = 1241.5x - 613$	0.50	1.60
Trimethoprim							
Plasma	0.4–20	7.0	4.4	2.0	$y = 8819.9x - 776$	0.15	0.30
Tissue	0.4–20	9.2	7.3	10.4	$y = 11394x - 599$	0.15	0.30
Linezolid							
Plasma	0.3–20	6.0	4.2	3.1	$y = 25946x - 3297$	0.15	0.30
Tissue	0.4–20	4.1	0.7	4.5	$y = 25940x - 3046$	0.15	0.30
Sulfamethoxazol							
Plasma	0.3–20	9.0	6.2	3.5	$y = 17107x + 2119$	0.15	0.30
Tissue	0.4–20	9.7	2.4	4.7	$y = 21576x - 1970$	0.15	0.30
Garenoxacin							
Plasma	0.4–10	2.0	9.0	3.0	$y = 16268x + 1320$	0.15	0.30
Tissue	0.4–10	15.0	10.0	7.0	$y = 19092x - 3900$	0.15	0.30

LOD, detection limit; LOQ, quantification limit; %R.S.D., relative standard deviation.

as the relationship between response and concentration. In this study, R.S.D.s resulted between 8.5% for TM and 15% for SMX. Table 3 shows the linear equations obtained for each antimicrobial where it is possible to see the low response obtained from AM compared with the other antimicrobials under evaluation. Limits of detection (LOD) as low as 0.1 μg/ml for AM have been reported using derivatization and a fluorescence detector [18]; however, LODs here are similar to those reported using UV detectors [10,11]. Considering the doses usually administered and levels reported in plasma and serum for the other analytes [10,11], the LODs found were satisfactory. Using fluorescence, the limits of detection for linezolid and sulfametoxazol were higher than those obtained for UV–vis. For this reason, the validation of the method with fluorescence was made only for garenoxacine. The LODs and LOQ were 0.03 and 0.06 μg/ml in plasma.

3.2.2. Precision and accuracy

The precisions both for the system and for the method in plasma and tissue were evaluated. Method precision was determined by using plasma and tissue sampled spiked and treated at three different concentration levels: low, medium and high. The R.S.D.s obtained for tissue were higher than those obtained for plasma. In all cases, R.S.D.s lower than 15% could be observed (Table 2). Recoveries at low, medium and high concentration were between 80% and 120% (Table 3), except for amoxicillin, which showed values of 71% and 78% for plasma and tissue, respectively, probably caused

Table 4
Accuracy for the method used for plasma and tissue treatment with acetonitrile

Antimicrobials	0.6 μg/ml %recovery	%R.S.D.	5 μg/ml %recovery	%R.S.D.	10 μg/ml %recovery	%R.S.D.
Amoxicillin						
Plasma	ND	ND	88 ± 5	11	71 ± 8	11
Tissue	ND	ND	85 ± 9	11	78 ± 8	10
Trimethoprim						
Plasma	118 ± 11	14	108 ± 16	15	114 ± 9	8
Tissue	104 ± 11	10	94 ± 2	2	104 ± 7	7
Linezolid						
Plasma	104 ± 4	3	89 ± 12	14	96 ± 5	5
Tissue	106 ± 13	14	92 ± 4	4	98 ± 5	5
Sulfamethoxazole						
Plasma	94 ± 10	11	108 ± 7	6	110 ± 12	11
Tissue	122 ± 14	11	102 ± 2	3	112 ± 7	6
Garenoxacin						
Plasma	111 ± 13	3	109 ± 11	10	107 ± 8	8
Tissue	93 ± 8	8	73 ± 6	7	85 ± 8	10

% R.S.D., relative standard deviation.

by a matrix effect. However, the %R.S.D.s were lower than 15%.

3.2.3. Stability

The fortified plasma samples could be stored at −20 °C for 6 months with no evidence of decomposition; treated samples at room temperature must be analyzed within 10 h of the treatment, because after 24 h the signals showed lower intensities by up to 50%.

3.2.4. Robustness

The assay for robustness involves the modification of several experimental variables to values higher and lower than the established conditions and further evaluation of some parameters in the obtained chromatogram. We used t_R as the evaluation parameter. The assay of robustness showed that the method is significantly affected by the flow rate (loss of resolution could be seen) and the solvent used for dissolution of the samples. Temperature and mobile phase affected the results to a lesser extent.

3.2.5. Selectivity

Peak identification in each chromatogram was achieved by means of t_R as well as spectral analysis. Analytical signals from both matrices (plasma and tissue) appeared at retention times different from the retention times of the antimicrobials; therefore, neither plasma nor infected tissues interfered with the analytes.

3.3. Applicability

To evaluate the effectiveness of the proposed method, plasma and tissue samples obtained from mice infected with *N. brasiliensis* were tested. Two mixtures were administered: mixture 1 consisted of TM, SMX and LZ and mixture 2 of LZ and GR. The combination SXT (SMX with TM) is the usual treatment for actinomycetoma [2]. However, this combination has to be administered over several months, even years, and no more than 70% of patients recover completely. For severe or resistant infections, amikacin is added to the mixture with a higher efficiency; however, some patients with resistant strains have been were reported. Amikacin, as with the other aminoglycosides, produce several secondary effects, such as ototoxicity and nephrotoxicity [2,3]. In recent studies, LZ showed a high *in vitro* effect against *N. brasiliensis* [4,5]. This antimicrobial drug has low toxicity and presents a unique action mechanism different to the SXT–amikacin mixture; therefore, the mixture SXT–LZ could be a new treatment scheme to treat with greater efficacy the patients with actinomycetoma.

The other mixture used was LZ–GR. These two antimicrobial agents present different mechanisms of action: while LZ

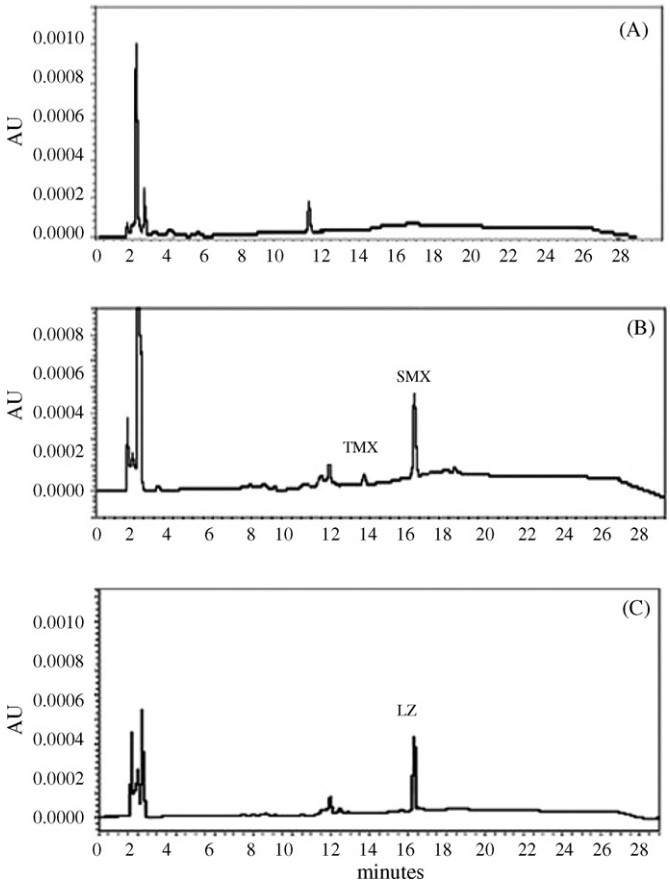

Fig. 3. HPLC chromatograms of plasma samples from a mouse treated with antimicrobials. (A) Plasma free of antimicrobials. (B) Plasma of a mouse treated with sulfamethoxazole–trimethoprim. (C) Plasma of a mouse treated with linezolid. Samples were taken 30 min after administration.

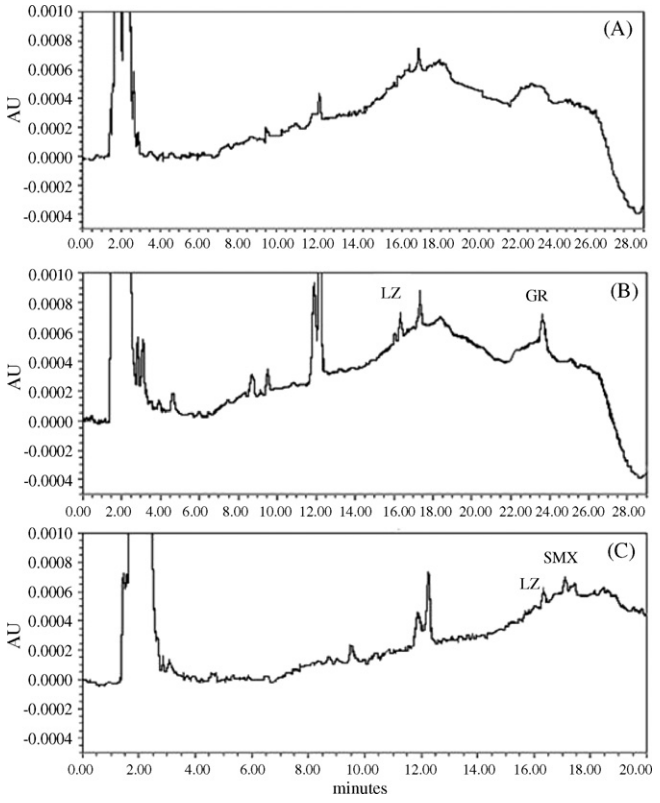

Fig. 4. HPLC chromatogram of infected tissue samples from mice treated with antimicrobials. (A) Tissue free of antimicrobials. (B) Tissue of a mouse treated with linezolid–garenoxacin. (C) Tissue of a mouse treated with linezolid–sulfamethoxazole–trimethoprim.

inhibits the protein synthesis, GR acts on DNA gyrase, an essential enzyme for the replication, transcription and reparation of bacterial DNA. The *in vitro* activity of GR against *N. brasiliensis* was recently probed by Vera Cabrera et al. This drug showed high activity, with an MIC_{90} of 0.5 μg/ml [6]. Taking this into account, the mixture LZ–GR could also be a good, low toxicity treatment for actinomycetoma.

Doses were selected taking into account the MICs reported for each antimicrobial against *N. brasiliensis* strains: TM, 4 mg/kg; SMX, 20 mg/kg; LZ, 25 mg/kg and GR, 75 mg/kg. These drugs were administered every 12 h over 3 weeks; after that time the mice were killed. Chromatograms obtained are shown in Figs. 3 and 4. Concentration of 4.06 μg/ml for LZ and 3.76 μg/ml for GR were found in the tissue samples by HPLC analysis.

These results show the applicability of the method, which can be applied in the future to obtain pharmacokinetic and pharmacodynamics parameters for these antibiotics.

This is the first report of a method for the simultaneous determination of amoxicillin, garenoxacine, trimethoprim, sulfamethoxazole and linezolid in plasma and tissue of actinomycetoma. The stationary phase used permitted the separation of all the antimicrobials without using the ionic pair reagent that has been referred to as the best analytical alternative for some of the compounds included in this work, such as garenoxacin and amoxicillin [12,14,15,25–27].

4. Conclusions

A method was developed and validated for the quantification of amoxicillin, trimethoprim, sulfamethoxazole, linezolid and garenoxacin in plasma and tissue from mice actinomycetoma. The HPLC method is easy to perform, and has adequate precision and accuracy. Because this study demonstrates the applicability of the method in a mouse model, it is reasonable to assume that it will be suitable for the development of *in vivo* studies using different treatment schemes to identify the best treatment for actinomycetoma on humans.

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