Quantification of Rifampicin and Rifabutin in Plasma of Tuberculosis Patients by Micellar Liquid Chromatography

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Abstract.

A Micellar Liquid Chromatographic procedure is described to determine Rifampicin and Rifabutin in plasma from Tuberculosis patients. Samples were diluted in mobile phase and then directly injected, avoiding long and tedious extraction steps. The analytes were resolved from the matrix without interferences from endogenous compounds using a mobile phase of sodium dodecyl sulfate 0.15 mol L-1 - 6%(v/v) 1-pentanol and phosphate buffer at pH 3, running at 1 mL min^{-1} through a C18 column at 25ºC. Detection was carried out by UV absorbance at 270 nm. Under these conditions, the final chromatographic analysis time was 22 min. The analytical methodology was validated following the FDA 2018 Bioanalytical Method Validation Guidance for Industry. The response of the drugs in plasma was linear in the 0.05-to-5 μg/mL range, with $r^2 > 0.9993$. Accuracy and precision were <14% for both substances. Carry over and matrix effects were negligible. Dilution integrity, robustness and stability were also investigated. Method was reliable, economic, eco-friendly, safe, easy-to-conduct, and with a high sample throughput, thus useful for routine analysis. Finally, the analytical method was used to determine both antituberculosis drugs in incurred plasma samples of Tuberculosis patients.

Keywords: Antituberculosis; Drug; Micellar; Optimization; Plasma; Patients.

1. Introduction

Tuberculosis remains a leading health issue worldwide and Rifampicin is the preferred first-line drug for its treatment [1-3]. Although it is quite well tolerated in a usual dose regime, adverse effects could be developed including gastrointestinal reactions, exanthema, hepatotoxicity and immunological reactions, as thrombocytopenia, leukopenia, eosinophilia, hemolytic anemia, agranulocytosis, vasculitis, acute interstitial nephritis and septic shock [4,5]. While some adverse effects may be resolved with symptomatic treatment or spontaneously, others may require regimen changes because they are dose-dependent [6]. On the other hand, Rifabutin has activity against Mycobacterium tuberculosis similar to Rifampicin, in fact in most of the cases may be more effective [7]. Main difference between them is that rifabutin has lower incidence of severe adverse effects [8,9]. This point makes Rifabutin more attractive as a substitute in situations where Rifampicin might cause adverse effects or is not well tolerated. Furthermore, Rifampicin has more drug interactions than Rifabutin due to it is a potent inducer of the CYP450 system [10]. Due to this fact rifabutin has been used in patients coinfected with tuberculosis and HIV [11], trying to avoid possible difficulties with drug interactions and avoiding the disease spreading between susceptible people [12]. On the other hand, Rifabutin is the only frontline antituberculosis drug that has activity against an emergent disease as Mycobacterium Abcessus, an opportunistic pathogen causing dangerous pulmonary infections because are intrinsically multidrug resistant [13].

Rifampicin (Fig. 1; log Po/w = 2.7 [14]) is the principal antituberculosis chemotherapy tool. However, Mycobacterium Tuberculosis develop resistance to this drug with high frequency restricting the utility of its use for treatment. Tuberculosis strains classified as multidrug-resistant (MDR) are those resistant at least to the two most potent first-line antituberculosis drugs, i.e. isoniazid and rifampicin [15-17]. The clinically significant resistance mechanism is mutation within a defined region of the rpoB gene, which encodes the target of RIF, the β subunit of bacterial RNA polymerase [18,19]. Most rifampicin-resistant Mycobacterium tuberculosis isolates are also resistant to rifapentine,

while approximately 15-20% of them are susceptible to rifabutin [20]. Although that confers a significant advantage, Rifabutin (Fig. 1; log Po/w = 4.1 [14]) to treat patients with MDR tuberculosis is limited by its high cost and the restricted availability [21].

Literature reveals several methods to determine this sort of compounds such as Liquid Chromatography (LC) and HPLC coupled with Mass Spectrometry (LC/MS) [22-25]. However, these methods require pre-steps on sample preparations, with several time-consuming and cumbersome extraction and purification steps due to complex chemical composition of plasma. Besides, they need an expensive specific instrumentation, a large amount of toxic solvents and qualified staff, which means increment price per analysis result.

To carry out the study, we use micellar liquid chromatography (MLC) as an analysis technique. Due to its advantages, MLC has been proven as a useful technique for routine analysis of a wide range group of drugs based on physicochemical properties of substances. MLC use hybrid mobile phases of sodium dodecyl sulfate (SDS) to drug determination in biological fluids. Micelles and monomers tend to bind proteins and other macromolecules competitively releasing bound drugs. Therefore, they are denatured and solubilized. In the column they are harmless and eluted at the front of the chromatogram instead of precipitating, allowing direct injection of the sample. Besides, the retention mechanism in the three-interaction environment is highly stable and reproducible, enabling its modeling by chemometrics. Another advantage is that the use of micellar mobile phases is nontoxic, non-flammable, biodegradable and relatively inexpensive if compared to aqueous-organic solvents [26,27].

The aim of the work is to develop a reliable, rapid, practical, easy-to-handle and inexpensive procedure to determine Rifampicin and Rifabutin in plasma using micellar liquid chromatography. Another goal is to demonstrate the advantages of the method and its possible establishment as a reference method of analysis. The method will be fully validated through FDA 2018 Bioanalytical Method Validation Guidance for Industry [28] to evaluate its analytical performance and used in incurred plasma samples to ensure its suitability for routine analysis.

2. Experimental

2.1 Reagents and chemicals

Rifampicin and Rifabutin (purity >99.6%) were supplied by Sigma (St. Louis, MO, USA). The other reagents used were sodium dodecyl sulphate (>99%) from Merck (Darmstadt, Germany), sodium dihydrogen phosphate (<99%), sodium acetate (>99.0%), HCl (37%), NaOH (>98%) from Panreac (Barcelona, Spain), and methanol, 1-propanol, 1-butanol and 1-pentanol (HPLC grade) from J.T. Baker (Deventer, Holland). Ultrapure water was in-lab produced using an ultrapure water generator device Simplicity UV (Millipore S.A.S., Molsheim, France), from deionized water, provided as tap water by the university. This water was used to prepare all the aqueous solutions.

2.2 Preparation of solutions

Micellar solutions were prepared by weighing in an analytical balance Metter-Toledo (Greifensee, Switzerland) the appropriate mass of SDS and salt buffer and solving them in ultrapure water with the aid of a magnetic stirrer. Afterwards, drops of HCl or NaOH were added to reach the desired pH, which was measured using a GLP 22 potentiometer equipped with a combined Ag/AgCl/glass electrode (Crison, Barcelona, Spain). Later, the proper volume was introduced to attain the sought proportion, and then the volumetric flask was filled up with ultrapure water. Finally, the solution was ultrasonicated in an ultrasonic bath Ultrasons-H (Selecta, Abrera, Spain), filtered through a 0.45-μm Nylon membrane (Micron Separations, Westboro, MA, USA) a 0.45 μm membrane filter (Micron Separations, Westboro, MA, USA) located in a Büchner funnel, with the help of a vacuum pump, and stored in an amber bottle.

Rifampicin and rifabutin stock solutions (100 mg L^{-1}) were prepared by solubilizing the appropriate amount in methanol, and the mixture was ultrasonicated for 2 min. Working solutions were prepared by successive dilutions in mobile phase, and renewed each month. All these solutions were stored in amber vials in a fridge at +4ºC. These solutions were thawed before use until complete solubilization of the SDS-crystals formed overnight.

2.3 Chromatographic conditions

The chromatograph used was an Agilent Technologies Series 1100 (Palo Alto, CA,

USA) equipped with a quaternary pump, a mobile phase degasifier, an autosampler with a 20-μL loop and a temperature controller for the column module coupled to a photodiode array detection. A Kromasil 5 C18 column with a 5 μm particle size and 150 mm (4.6mm i.d.) (Scharlau, Barcelona) was used. HP ChemStation Rev. B.01.01 software was used to control the instrumentation, as well as to monitor, acquire and process the chromatographic signals. The dead time ($t_0 \approx 1.0$ min) and the retention time (t_R, min) were directly taken from the chromarogram, while retention factor (*k*), efficiency (*N*, number of theoretical plates), asymmetry factor (*T*), peak width at half-height ($w_{0.5}$, *min*) and resolution (*Rs*) was calculated as in [29]. Working and cleaning instructions about the chromatographic instrumentation when dealing with micellar solutions is described in [30].

The mobile phase was and aqueous solution of 0.15 mol L^{-1} SDS/6%(v/v) 1-pentanol/phosphate buffer at pH 3, running at a constant flow of 1.0 mL min^{-1} at 25ºC. Under these conditions, the Kromasil C18 column head pressure was around 150 bars. The detection absorbance wavelength was 270 nm. Solutions were filtered through a 0.45 μm membrane filter by pushing with a 3-mL syringe.

2.4 Sample collection and treatment

Incurred plasma samples were obtained from tuberculosis patients following rifampicin/rifabutin therapy, while blank samples from healthy volunteers not taking any medication. All of them were extracted by qualified medical staff and provided by a local Hospital. The investigation was approved by two local Ethics Committees, Hospital and the University Ethic Committee for Analysis of Research Projects. Written informed consent was obtained from all participants and all research was performed in accordance with the 2013 Helsinki Declaration principles.

For confidentiality reasons, samples were sent unlabeled. Neither personal nor clinical information about the patients or the healthy volunteers was provided from the Hospital. The laboratory undertakes not to transmit any information to other institutions (except that indicated in the paper). Besides, the laboratory commits to destroy all the samples (except those from healthy volunteers, which may be stored from further studies) and all the chromatograms and the experimental data (except that here published) one year after the

publication of the paper.

Blood samples were collected using a DB SST Tube (BD Vacutainer Systems, Plymouth, UK), and were centrifuged for 5 min at 756 Relative Centrifugal Force (RCF) or G-force at 4ºC, to obtain the non-cellular fraction. Plasma was immediately frozen and kept at −20°C. Plasma samples, either blank or patient samples, were processed as the same way. They were thawed until complete melting the same day of the analysis. Afterwards, an aliquot was 1/5-dissolved in mobile phase, filtered and directly injected. The fortification was performed by adding the appropriate volume of a standard solution of the analytes, before the dilution [26,27].

3. Results and discussion

3.1 Optimization of chromatographic conditions

The simultaneous separation and quantification of the analytes within the minimum analysis time and the maximum resolution and efficiency are the main objectives in the development of the MLC method for determination of solutes in biological samples. Thus, an optimization strategy might be considerable helpful to find the best chromatographic conditions. The optimization strategy may be sequential or interpretative [26]. Sequential strategies have proved to have shortcomings when several local and/or secondary maxima exist; in fact, the optimum spot did not always correspond to the best maximum. Thus, the interpretative strategy may be much more efficient and reliable. Moreover, the interpretative strategy allows taking into account independent variables. In the interpretative strategy the experiments are designed before the optimization process and used to fit a model that allows prediction of the parameters in a wide range of space. An interpretative strategy was chosen for use in the present work.

One of the interpretative strategies is called factorial experiment design and is based on varying all factors simultaneously at a limited number of factor levels. This kind of experimentation is especially important at the beginning of experimental study, where the most influential factors, their ranges of influence and factor interactions are not yet known. Factorial experiments allow experiments to take place over the whole range of the factor space. They show a high degree of precision in exchange for a minimum experimental effort

and they enable factor interactions to be detected. Optimization studies were performed using a working standard solution of 0.2 mg L^{-1}.

3.1.1 Parameters selection

The mobile phase selection was based on the resolution and a suitable analysis time of the two compounds, Rifampicin and Rifabutin, including a third peak corresponding to the band of proteins eluted at lower time. The octanol–water partition coefficient (logPo/w) of Rifampicin and Rifabutin are 2.7 and 4.1 respectively, which means that it is hydrophobic in nature and can be expected to have high retention in MLC.

In the development of the MLC method for the determination of Rifampicin and Rifabutin, eight factors were identified that influence the separation analytes: SDS and modifier concentrations, type of buffering species, running buffer pH, column length, column temperature, sample injection volume and flow rate. The criteria for the selection of the optimum separation conditions were the maximum separation of the analytes within the minimum analysis time. Thus, peak width (i.e., resolution) and retention time were parameters of primary importance to assess the goodness of the experimental result. On the other hand, detection wavelength was optimized to get maximum signal-to-noise ratio.

First of all, we studied the effect of the pH on the chromatographic response over the working range of the column: 3.0; 7.0 (phosphate buffer) and 5.0 (acetate buffer). Under these conditions, the formal charge of Rifampicin and Rifabutin are +1.2; +1.0; 0 and +2; +1.2; and +0.5, respectively [31]. For rifampicin, retention time was similar at the three pHs, while rifabutin was eluted earlier at 3.0. Resolution was adequate in all cases. Therefore, pH 3.0 was selected. Second, molarity of the buffering agent was observed to have less influence on the separation and was therefore kept constant above 0.01 M. Third, the column length was studied making efficiency increase a 10% in a column of 25 cm compared to one of 15 cm, but retention time and analysis increased in a 35%, so a column of 15 cm was selected. Fourth, the influence of the temperature was checked in the range from 25 to 40ºC, result shows that this parameter does not improve the resolution or analysis time: 25ºC was selected for further studies. Fifth, the studies were focused in the injection volume, changing it between 0.5-30 µL, observing overloading at 25 µL: an injection volume of 20 µL was selected. Sixth, flow rate was experimented from 0.5 to 2 mL min^{-1} showing a decrease of

analysis time, but decreasing the resolution dramatically, so 1 mL min^{-1} was selected. Finally, SDS and modifier concentrations have an important influence and were optimized using a factorial design.

Methanol, 1-propanol, 1-butanol and 1-pentanol are the most used short-chain alcohols as modifiers. All of them were checked noting that Rifabutin is more susceptible to modifier concentration. Using 1-pentanol acts reducing retention time in more than hour in Rifabutin and more than fifteen minutes in Rifampicin compared to methanol. As a retention time is a priority parameter, 1-pentanol was selected as the modifier for the separation of Rifampicin and Rifabutin. Also, the use of 1-pentanol improves resolution peak, significantly.

3.1.2 Mobile phase selection using factorial designs

The effects of SDS concentrations in the range 0.05 to 0.15 mol L^{-1} and 1-pentanol in the range 2 to 6% (v/v) were selected [32]. Low values of both parameters could produce sub-micellar media, and upper the maximum level, microemulsions [33].

To evaluate the influence of SDS and 1-pentanol on the separation we used a three-level full factorial design (3^2). The parameter settings and the design are reproduced in Table 1.The runs of the design were carried out in a randomized sequence and the retention times and peak widths were measured. Varying all factors simultaneously at a limited number of factor levels, and after the calculation of the function responses, a polynomial curve was obtained. As responses, two different functions were checked: first, the product of resolutions and, second, the chromatographic resolution statistic (CRS) function [34]. The equations of the model are in Table 2.

Table 1. 3^2 factorial design and obtained responses.

Run	[SDS] (M)	[1-pentanol] (%, v/v)	Resolution	1/CRS
1	0.1	6	152.3	3.43
2	0.05	4	87.4	2.17
3	0.1	2	31.4	1.05
4	0.15	2	32.2	1.01
5	0.15.	6	152.4	3.47

6	0.05	6	149.8	3.35
7	0.1	4	87.2	2.15
8	0.05	2	32.9	1.04
9	0.15	4	87.6	2.28

Table 2. Equations used for the statistical model

Equation number	Determined parameters	Equation
(1)	Resolution of a pair of peak ($R_{o;\,o+1} = R_i$)	$R_{o+1;o} = \dfrac{1.18\ (t_{o+1} - t_o)}{(w0.5_{o+1} + w0.5_o)}$
(2)	Total resolution (Rs)	$Rs = \prod_{o=1}^{o=n-1} R_{o;o+1} = \sum_{i=1}^{i=n-1} R_i$
(3)	General equation relating the response to the factors	$y = b_0 + \sum_{a=1}^{c} b_a x_a + \sum_{1 \le a \le b}^{c} b_{ab} x_a x_b + \sum_{a=1}^{c} b_{aa} x_a^2$
(4)	Chromatographic resolution statistic (CRS)	$CRS = \left\{ \sum_{o=1}^{n-1} \left[\dfrac{(R_{i,i+1} - R_{opt})^2}{(R_{i,i+1} - R_{min})^2 R_{i;i+1}} \right] + \sum_{i=1}^{n-1} \dfrac{R_{i;i+1}^2}{(n-1) R_{av}^2} \right\} \dfrac{t_n}{n}$

Meaning of the subscripts:

o, compound eluting at o^{th} position (in increasing order of retention time)

i, resolution of between the i^{th} pair of peaks (i = o)

n, number of analytes; the number of paired peaks is n-1

c: number of factors; a,b: two factors; b_0, intercept parameter, and b_a, b_{ab}, and b_{aa}, regression parameters for linear, interaction, and quadratic factor effects, respectively.

$R_{i, i+1}$: resolution between consecutive peaks

Rav: the average resolution of all peaks

Rmin the minimum acceptable resolution

Ropt: is the desired (optimum) resolution

t_n is the retention time of the last-eluting solute

The resolution (Ro; o+1) of a pair of peaks was calculated using Eq. (1). The numerator in Eq. (1) describes the separation process with regard to differential retention and the denominator expresses the dispersive processes acting against it. The total resolution (Rs) was set as response and calculated as the product of the resolutions of the all pairs of peaks (eq. 2). To quantify and interpret the relationships between responses and factor effects a response surface methodology was used. The general empirical model is a second-order polynomial, where the response y is related to the variables (factors) x as indicated in eq. (3). The nonlinear regression analysis of the data was carried out using the SPSS 20.0 software (SPSS Inc., Chicago, IL, USA. The model obtained was:

$$Rs = - 12.32 – 6.14\ [SDS] + 20.65\ [pentanol] + 2.04\ [SDS]^2 + 1.05\ [pentanol]^2 – 0.07\ [SDS]\ [pentanol] – 1.01\ [SDS]^2\ [pentanol] + 0.55\ [SDS]\ [pentanol]^2$$

Where [SDS] is in mol L^{-1} and [1-pentanol] in %, v/v. Results at the assayed conditions are in Table 1. The three-dimensional plot of total Rs, as a function of SDS and 1-pentanol, is shown in Fig. 2A. The surface plot allows the whole range of conditions to be explored, including combinations that were not experimentally demonstrated, indicating that the maximum resolution area corresponds to 0.15 mol L^{-1} and to a 1-pentanol concentration of 6% (v/v). Thus, these conditions were considered to be the optimum MLC conditions to separate the two antituberculosis drugs, including the band of proteins in the plasma samples.

The same optimum was achieved when a second response function is used, which is the inverse of the chromatographic resolution statistic. The CRS is a mathematical function calculated with Eq. (5). Rmin was set to 1. Considering that peak width is 1 min, two peaks, which appear contiguous, have a difference in time of 1 min. Thus, Rs calculus shows that the result is 1 (minimum acceptable separation). Ropt was 1.5. Rs is 1.5 if the two peaks are separated by a time of 0.5 min. The CRS considers the resolution of all solutes in the sample and incorporates three important aspects of the separation. The first term in Eq. (5), named

the resolution term, evaluates the resolution between all adjacent solute pairs in comparison to defined values for optimum and minimum resolution. The second term in Eq. (5), named the distribution term, considers the relative spacing of the solute zones. The final multiplier term in Eq. (5) takes into consideration the analysis time and the number of analyte peaks to be separated. The inverse of CRS was chosen because the maximum of the function fits the optimal condition. The response surface of this response function (inverse of CRS) was compared with the results obtained with the Rs function Results at the tested conditions are in Table 1. Fig. 2B shows the surface plot and the maximum of this function coincided with the optimum conditions obtained with the Rs function. The mathematical model obtained was Eq. (5).

$$CRS^{-1} = - 0.08 + 0.01\ [SDS] + 0.54\ [pentanol] - 0.003\ [SDS]^2 + 0.002\ [pentanol]^2 - 0.004\ [SDS]\ [pentanol] + 0.006\ [SDS]^2\ [pentanol] + 0.04\ [SDS]\ [pentanol]^2$$

To summarize, the conditions selected were as follows: SDS 0.15 M-pentanol 6%-$H_2PO_4^-$ 0.01 mol L^{-1} at pH 3 in a C18 column of 15 cm thermostatized at 25ºC, with 20 µL of injection, and a flow rate of 1 mL min^{-1}. A system suitability test (SST) was performed by the replicate injection (n=6) of working standard solution of 0.2 mg L^{-1} (Table 3) [37]. Instrumentation and experimental conditions were valid for analysis, as the responses comply with the acceptance criteria. Additionally, spectra were taken at the retention time, 50%- and 5%-leading and tailing edges.

Table 3. Results of the system suitability test

Parameter	Rifampicin	Rifabutin	Acceptance criteria
t_R (min) (RSD, %)	5.88±0.05 (0.9)	18.1±0.1 (0.6)	RSD <1%
RSD of peak area	0.8	0.6	<1%
RSD of $w_{0.5}$	0.9	0.7	<1%
retention factor	5.9	18.1	>2.0
Efficiency	3551	2974	>2000
Asymmetry	1.2	1.1	0.8-1.6

t_0 = 1.0 min

3.2 Method validation

This methodology for the determination of Rifampicin and Rifabutin in plasma of Tuberculosis patients has been validated according to the FDA 2018 Bioanalytical Method Validation Guidance for Industry and other documents about validation [35,36] which includes selectivity, linearity, calibration range, sensitivity, limits of detection (LOD) and quantification (LOQ), carry-over, trueness, precision, dilution integrity, stability, intra- and inter-day precisions, selectivity, recovery and robustness. All the parameters were determined in matrix, otherwise specified.

3.2.1 Selectivity

To study the matrix effects in plasma of the possible co-eluting compounds, ten blank samples were analyzed (Fig. 3A). The protein band appeared from dead time to 2.5 min, the baseline at longer times was quite stable, and there were no peaks near or at the window time of the analytes. Blank plasma sample spiked with 1 μg/mL of Rifampicin and Rifabutin were analyzed; and the peaks of the two analytes are sufficiently separated between them. Overlapping was not detected either among them, or with the front of the chromatogram or with other compounds (Fig. 3B). Chromatogram shape was similar to those obtained by the analysis of the blanks. Peaks exhibited similar retention time, area and profile than in 3.1.2.

A peak-purity study was performed. UV absorbance spectra were taken at the same points that in 3.1.4; and compared to that there-obtained by overlaying and visual observation. No significant difference was observed, thus pointing to the absence of coeluting compounds.

3.2.2 Calibration range, linearity and sensitivity

Blank samples were spiked at nine concentration levels of Rifampicin and Rifabutin in the range 0.05-5 mg L^{-1}, and analyzed by triplicate. For each analyte, the variance of the peak area was found similar for all the concentration values, and then the data can be considered homoscedastic. A correlation between the average values of the chromatographic peak area (response) versus the concentration (independent variable) was obtained by least square linear regression. Results were:

Rifampicin: A= (222 ± 15) [RIFAMPICIN] - (2.6 ± 2.50), determination coefficient (r^2)=

0.9994; relative residual standard deviation (RRSD,: 3.2%

Rifabutin: A= (388 ± 3) [RIFABUTIN] - (2 ± 5), r^2 = 0.9993; relative residual standard deviation: 1.8%

An adequate goodness-of-fit was obtained, as r^2 and RRSD were >0.99 and <5%, respectively; and the plot residual *v.s.* concentration did not show a trend. No outliers were found, as the relative residuals and the Cook's squared distance (CD^2) were <3 and <1, respectively. No systematic error was noticed, as the y-intercept confidence interval included 0.

The limit of detection was set to 3.3 times the standard deviation of the blank (that of the y-intercept) divided by the slope: rifampicin, 0.03 mg L^{-1} and rifabutin, 0.01 mg L^{-1}. Lower limit of quantification (LLOQ) was the minimal concentration complying with the accuracy/precision acceptance criteria, 0.05 mg L^{-1}. The upper limit of quantificatrion (ULOQ) was set to 5 mg L^{-1}.

3.2.3 Precision and trueness

These parameters were within and between-run determined at four levels: 0.05; 0.15; 1 and 2.5 mg L^{-1}.

Within-run parameters were determined by the successive analysis of six fortified samples, placed in the same sequence run. Accuracy was quotient between the average of the found concentration and the true one, while the precision was the relative standard deviation (RSD) of the found concentration. To find out the between-run values, the same experiment was repeated five times on different days over a 3-month period, using renewed spiked samples with renewed standard working solutions. Accuracy was the ratio average of the five average found concentration/spiked one, while precision was the RSD of the five found concentrations. Results can be seen in Table 4. For both analytes, accuracy (86.0-108.7%) and precision (<8.5%) fit the acceptance criteria, and then the method provides straightforward quantitative data. This performance was attained mainly since the sample is quantitatively introduced on the sample without extraction.

Table 4. Within-run and between-run accuracy and precision (^{a}n=6; ^{b}n = 5). Acceptance criteria: accuracy 80-120% for LLOQ and 85-115 for higher values; precision, for LLOQ

<20% and for higher values, <15%.

	Rifampicin			
	Within-run[a]		Between-run[b]	
Concentration	Precision	Accuracy	Precision	Accuracy
0.05	7.4	86.0	8.5	88.9
0.15	6.4	89.8	7.0	91.5
1	4.2	95.4	3.5	97.5
2.5	1.3	98.9	1.9	99.4
30 (1/10 dil.)	4.2	91.6	5.8	96.8
	Rifabutin			
Concentration	Precision	Accuracy	Precision	Accuracy
0.05	7.0	108.7	7.9	106.2
0.15	6.8	105.2	6.0	105.9
1	3.5	98.6	3.0	103.4
2.5	2.0	101.5	2.5	98.7
30 (1/10 dil.)	4.6	102.9	6.1	99.2

3.2.4 Carry-Over Effect

A plasma sample spiked with Rifampicin and Rifabutin at 5 μg/mL was injected, and immediately afterwards, a blank plasma sample was analyzed. In this last one, no peak was observed in the chromatogram at Rifampicin or Rifabutin retention time. Thus, the carry-over was considered negligible at concentrations within the calibration range.

3.2.5 Dilution Integrity

The effect of the introduction of another dilution step was investigated. Plasma samples spiked at 30 mg L^{-1} were 1/10 diluted in mobile phase and then processed as in Section 2.4. The inter- and intra-day accuracy and precision were determined as in 3.2.3 (Table 4). The results were inside the acceptance criteria, and the method allows a sample over ULOQ to be analyzed by the proper dilution.

3.2.6 Matrix Effects

The influence of the endogenous compounds of plasma in the quantitative results, either by linking or any interaction interfering with the retention process, was evaluated. A calibration curve was performed using working standard solutions containing the same concentration of Rifampicin and Rifabutin as in Section 3.2.2, divided by five (to consider the dilution in the sample treatment). The slopes were similar to that obtained in 3.2.2, pointing to the absence of matrix effect. This was due to the preferential interaction of SDS-monomers and micelles with proteins and macromolecules of the biological fluids, thus inhibiting their binding with the drugs.

3.2.7 Robustness

The variation of the main chromatographic responses (retention time and peak area) were examined at small, but deliberate, changes in the main operational parameters. These were changed from their optimal value, and the tested oscillations were that we considered that may occur during the laboratory work and chromatographic run, in a usual situation. The studied factors and the corresponding interval were: A) detection wavelength ± 5 nm; B) SDS in mobile phase ± 0.05 M; C) 1-pentanol in mobile phase ± 0.2%; D) pH in mobile phase ± 0.2; E) flow-rate ± 0.05 mL min^{-1} and F) injection volume ± 2 μL. A blank plasma sample spiked with 1 mg L^{-1} was analyzed by testing eight different set of experimental conditions, which value was fixed by an experimental design following a Youden approach [37]. Results can be seen in Table 2. Differences > 8.0% were judged significant, meaning that they may be especially controlled during the analysis. For both compounds, the retention time was significantly affected by the low-rate, while peak area by the injection volume. Besides, for rifabutin, SDS concentration influences the retention time. Therefore, these factors operational parameters must be especially controlled during analysis to achieve adequate analytical results.

3.2.8 Stability

Stability means the capacity of the drug to remain unchanged throughout time. It can

be studied under different environmental conditions of light, temperature, chemical environment – among others. It is determined by the monitoring of the concentration of analyte in a stored solution or sample. The degradation is noticed by the diminishing of the concentration and the emergence of peaks from decomposition products through time. As reported in the literature, rifampicin is a highly unstable drug and readily decomposes in Rifampicin quinone due to oxidation [38] while Rifabutin is quite stable; being more affected by thermal and light conditions [39].

Stability of Rifampicin under the below mentioned environments has been largely studied in [30]. In the frame of this research, we investigated the decay of rifabutin under the following conditions:

a) Working solution in mobile phase (0.2 mg L^{-1}): the solution was analyzed fresh, and then stored at -4ºC. Each day for one week, the solution was thawed, analyzed and reintroduced in the fridge. No significant decrease in concentration was observed (acceptance criteria 5%). Therefore, working solutions were renewed after this period.

b) Bench-top plus autosampler of processed sample at room temperature for 1 day: a treated 1-mg L^{-1}-fortified plasma sample was placed in the autosampler tray and analyzed by successive injections for 1 day. No decomposition was perceived (acceptance criteria 15%), and then a sample can be analyzed until 1 day after processing, without affecting the reliability of the method.

c) Long-term stability of plasma sample spiked at 1 mg L^{-1} at the usual storage conditions and time in a Hospital: A plasma sample spiked was prepared, analyzed (fresh), divided into 15 parts and kept at -20ºC. Thereafter, each day, one of them was thawed, analyzed and discarded. The drug remain quite stable for the duration of the assay (acceptance criteria 15%), and then a plasma extract can be kept for two weeks under these conditions without reducing the accuracy of the quantitative result.

3.3 Application of the method to patient´s plasma

The method was applied to incurred plasma samples from tuberculosis patients taking a medication based on the studied drugs. Quality control samples (QC), blanks, and incurred sample reanalysis (ISR) were included in the same run. Samples were firstly processed and then put in the autosampler. Results can be seen in Table 5. Chromatogram obtained from

patient M1 can be visualized in Figure 3C.

In all cases, rifampicin and rifabutin were visualized without interferences. No drift was noticed, and the quantitative data remain quite consistent, since the QC comply with the accuracy criteria and the ISR were <20% of their average. The entire set (34 injections) was analyzed in a single day by one operator, despite of the large number of samples, thus proving a high sample throughput. Only generic reagent, material and instrumentation were used. Volume of toxic chemicals handled and wasted (only the 6% of 1-pentanol in the mobile phase) was quite low. The cost of the determination per sample was minimal. Therefore, the method exhibited strong practical performances and is useful for routine analysis in laboratories with a high workload, like a clinical one.

Table 5. Analysis of incurred samples

Sample	Rifampicin	Rifabutin	Sample	Rifampicin	Rifabutin
Blank	n.d.	n.d.	M10	3.84	3.47
M1	1.85	1.39	M11	0.54	0.89
M2	0.61	0.44	0.05	0.06	0.05
0.05	0.05	0.06	M12	Under LOQ	n.d.
M3	0.09	0.14	M10-bis	3.74	3.32
M4	5.31	4.22	M13	1.78	1.44
0.015	0.016	0.017	0.15	0.014	0.015
M5	0.09	0.15	M14	4.75	5.94
M6	0.39	0.28	M15	1.58	1.29
1	1.08	1.05	1	0.97	0.95
M6	2.83	2.31	M16	3.25	3.09
M4-bis	5.15	4.32	M15-bis	1.54	1.23
M7	0.08	0.06	M17	0.08	0.10
2.5	2.54	2.56	2.5	2.47	2.51
M8	n.d.	n.d.	M18	3.85	4.82
M9	0.81	0.75	M19	0.78	0.95
Blank	n.d.	n.d.	M20	1.74	2.32

4. Conclusions

Micellar liquid chromatography has been proved as a suitable technique to analyse

Rifampicin and Rifabutin in human plasma. One advantage of this procedure is the possibility of injecting diluted sample into the chromatographic system, avoiding long and tedious extractions. After sample irradiation to improve sensitivity, the analyte was satisfactorily resolved using a mobile phase of 0.15 mol L^{-1} SDS-6%(v/v) 1-pentanol/phosphate buffer at pH 3 from the matrix in an analysis time of under 20 min. Validation was performed according to the FDA 2018 Bioanalytical Method Validation Guidance for Industry with satisfactory results in terms of linearity, selectivity, precision, accuracy, carry over, dilution integrity, matrix effect, robustness and stability. The limit of detection and the lineal range were sufficient to detect the usual amount of Rifampicin and Rifabutin in patient´s plasma. Also, stability studies could be performed using the purposed method. Moreover, this method is relatively inexpensive, sustainable, and exhibit a high sample throughput, which make it as an excellent alternative in a clinical laboratory.

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6. Conflicts of interest

The authors declare there are no conflicts of interest.

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FIGURE CAPTIONS

Figure 1. Chemical structure of Rifampicin and Rifabutin

Figure 2. Three-dimensional response surface of (A) resolution and (B) 1/CRS as a function of the concentration of SDS and 1-pentanol in the mobile phase.

Figure 3. Chromatogram obtained by the analysis of a plasma sample: A) blank; fortified at 1 mg L^{-1} of booth drugs, and C) from patient M1.