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Stability studies of rifampicin in plasma and urine of tuberculosis patients according to the European Medicines Agency Guidelines

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Abstract

Aim: The macrolide antibiotic rifampicin is prescribed against several infections, like tuberculosis disease. This drug decays to rifampicin quinone.

Results/methodology: The biological fluids were diluted in a micellar solution and directly injected. Using a C18 column and a mobile phase of 0.15 M SDS-6% 1-pentanol phosphate-buffered at pH 7, running at 1 ml/min, the analytes were resolved in less than 15 min. The detection was by absorbance at 337 nm. Method was validated by the guidelines of the European Medicines Agency. Decomposition of rifampicin to rifampicin quinone was also studied.

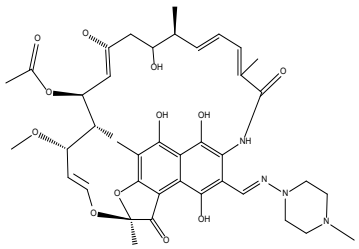
Discussion/conclusion: Procedure is rapid, easy-to-handle, economic, eco-friendly and with a high sample throughput. It was successfully used to monitor rifampicin in the plasma and urine of tubercular patients.

Keywords: Plasma and Urine Samples; Rifampicin; Stability studies; Tuberculosis; Validation.

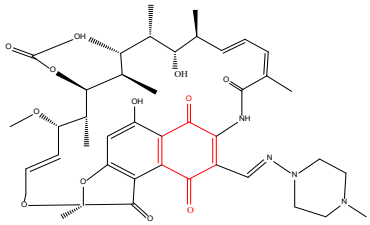
Introduction

Rifampicin is a semi-synthetic macrocyclic antibiotic Figure 1A [1], lipophilic substance with a log Po/w value of 4.24 and pKa of 1.7 [2]. The stability of the drug mainly depends on the pH of the medium. It has been reported that in neutral pH the drug is stable whereas in acidic and basic pH it is unstable. In acidic media, rifampicin is hydrolysed to produce 3-formyl rifampicin which is further oxidized to form an inactive quinone derivative i.e. rifampicin quinone (Figure 1B) [3]. Thus rifampicin either pure or in formulations is an immunosuppressant [4] and is used in tuberculosis, leprosy and legionnaire's disease treatment, but when is stored suffers oxidative decomposition to produce inactive rifampicin quinone.

Tuberculosis (TB) is a chronic infectious disease which is caused by a microorganism of the genus mycobacterium. It spreads when a healthy person comes in contact with a person who is infected with these bacteria. In case proper medication is given to the infected tuberculosis patients regularly and properly, this disease is completely curable. WHO recommends a four drug combination: rifampicin, isoniazid, ethambutol and pyrazinamide, as the first line anti-tuberculosis drug [5-7]. The entire treatment period is of six months which is sub-divided into two phases of four months and two months respectively. In the first phase all the four drugs are given to the patient and in the second phase out of the four prescribed drugs only rifampicin and isoniazid are given daily [7]. A single dose of 600 mg rifampicin is capable of killing 99.9% or more of viable organisms. According to the published facts, rifampicin may exert a delayed antibiotic effect for several days, during which the organism multiplication is inhibited [8]. On the other hand, adverse effects of rifampicin includes nausea, vomiting, stomach pain, heartburn, diarrhoea, muscle or joint pain, headache, rash or itching, drowsiness, dizziness, spinning sensation or numbness or tingling in legs [9]. Due to above-mentioned side effects patients stop taking their regular dose of medicine. If so, the patients tend to pass to be multidrug resistent patients. Thus there is an urgent need for the monitorization of patients who undergo treatment at the Indian Program Directly Observed Short Course (DOTS) center.



(A)



(B)

Figure 1. Structures of Rifampicin and Rifampicin quinone.

There are several analytical methods reported by researchers for the determination of rifampicin and its oxidative product in different matrices. The spectroscopic methods have been widely used for the determination of rifampicin and are one of the most common and frequently used analytical techniques [10,11]. As the spectroscopic methods are known for poor selectivity, thus there is a need for hyphenated separation technique. For the separation of isoniazid, ethambutol, rifampicin, and pyrazinamide in fixed dose combination an advanced form of planar chromatography i.e. thin layer chromatography (TLC) [12], high performance thin layer chromatography (HPTLC) was used [13]. Researchers had also developed a stability indicating [14] and densitometric HPTLC method for the simultaneous determination of rifampicin and isoniazid in rat plasma [15]. HPTLC is still considered to be a screening technique and for the purpose of confirmation and quantitative analysis, high-performance liquid chromatography (HPLC) remains to be the method of choice. As reported by different groups HPLC is commonly used for the determination of the antitubercular drugs in various matrices, i.e. in formulations, for the purpose of quality control [16] as well as in biological samples for therapeutic drug monitoring [17]. Another

reported technique for the simultaneous determination of the anti-tubercular substances in various formulations is by capillary zone electrophoresis (CZE) which is also a useful separation technique [18]. Many research groups has also reported the use of LC-MS/MS but the method is quite expensive [19-20] as well as the technique is not readily available in some places of India. The main drawback with all the reported methods is the time of analysis and the fact that sample preparation is tedious as well as all of the developed techniques required extraction of the drug from blood and other biological matrices [21-22].

Micellar liquid chromatography (MLC), using sodium dodecyl sulphate (SDS) as the surfactant and a C18 column, has been proven as an interesting alternative to hydro-organic RP-HPLC to resolve drug mixtures in plasma [23-27]. Micelles interact with the proteins of serum and urine, provoking their denaturation, solubilization and the release of bounded drugs. Moreover, solubilized matrix substances are barely retained in the C18-column. Therefore, plasma and urine samples, after a dilution with a micellar solution and filtration, can be directly injected, as the matrix is harmless eluted at the front of the chromatogram, thus expediting the experimental protocol [28]. The presence of the micelle pseudo phase and the modification of the stationary phase by the SDS monomer increase the number of interactions inside the column for the analytes, which complexes the retention mechanism. Besides it only requires the addition of a small amount of short-chain alcohol (up to 12.5 %) to improve the resolution [29]. This makes MLC a highly versatile technique and able to resolve mixtures of drugs in a wide range of hydrophobicity using an isocratic mode.

The aim of the work is to develop a reliable analytical procedure based on MLC to determine rifampicin in plasma and urine and for studies of its stability. The method should be inexpensive, simple, eco- friendly and enough sensitive to detect the usual concentrations of these drugs in plasma and urine from tuberculosis patients following a therapy. The method was validated by following the guidelines of the European Medicine Agency (EMA) [30]. The developed method

was applied to study the presence of rifampicin in plasma and urine samples of tuberculosis patients and also to decomposition studies to rifampicin to rifampicin quinone.

Experimental

Standard and reagents

Standard of rifampicin and rifampicin quinone (purity > 99.0%) was supplied as gift by Wilcure (Wilcure Remedies Private Limited, Indore, India) which is a quality control pharmaceutical company. All other chemicals used to prepare mobile phase as SDS (purity>99.0%) were purchased from Merck (Mumbai, India), sodium dihydrogen phosphate, hydrochloric acid and sodium hydroxide were purchased from Himedia Laboratories Private Limited, (Mumbai, India). HPLC grade water, propanol, pentanol, butanol, methanol was purchased from Rankem (Ranem RFCL Limited, New Delhi, India). All chemicals used were of analytical grade. Double distilled water was prepared using a Borosil Distillation Unit (Borosil Glass Works Limited, Mumbai, India) and all the solution were filtered through 0.45 μm nylon membrane, Micron Separation, (Westboro, MA, USA).

Apparatus and instrumentation

A Metter-Toledo analytical balance (Mettler Toledo India Private Limited, New Delhi, India) was used to weigh the analytes. The pH was measured with a pH-102/103 Contect (Instruments Limited, Mumbai, India) equipped with a combined Ag/AgCl/glass electrode. An ultrasonic bath model PCI (Analytics Private Limited, Mumbai, India) was used to dissolve the standards.

A chromatographic system Shimadzu Prominence HPLC (Shimadzu Corporation, Kyoto, Japan), equipped with an isocratic pump LC-20 AT, an auto-sampler SIL-20AC, and a diode array detector SPD-M20 A (190-800 nm) was used. The separation was carried out on SPHER-100 C18

100A (250 mm x 4.6 mm, 5 µm particle size) from Princeton Chromatography INC, (Cranbury, NJ, USA). Chromatographic signals obtained, were processes on a personal computer which was connected to the instrument using LC Solution version 1.22 SP1 software (Shimadzu Corporation Kyoto, Japan). Other statistical calculations were performed using Microsoft Office Excel 7 (Microsoft Corporation, Seattle, WA, USA) and Origin pro 8 (Northampton, Massachusetts, USA). For the selection of wavelength, an UV-Vis Systronic double beam 2201 spectrophotometer was used (Ahemdabad, India). The incurred samples were centrifuged using Compact cooling centrifuge CM-8 plus, Remi electrotechnik limited (New Delhi, India) and kept in deepfreezer (Remi Electrotechnik Limited, Mumbai, India). The experimental chromatograms were treated using the Michrom Software [31], to determine the chromatographic parameters: dead time (t_0) retention time (t_R), efficiency (N), assymmetry factor (B/A) and peak area under the curve (AUC).

Solutions and mobile phases

Stock solution of rifampicin and rifampicin quinone (100 µg/mL) was prepared by weighing the appropriate amount of standard and dissolving in 5 ml of methanol, completing with a micellar solution of 0.15 M SDS, 1-pentanol 6% (*v/v*), pH 7. Working solutions were made by successive dilution of these stock solutions with the solution of 0.15 M SDS, 1-pentanol 6% (*v/v*), pH 7. These solutions were renewed each four days.

Micellar solutions were prepared by weighing the appropriate amount of SDS and 0.01 M sodium dihydrogen phosphate, and then dissolved in double distilled water. The pH was adjusted to the desired value by adding drops of 1.0 M hydrochloric acid or 1.0 M sodium hydroxide solutions. The adequate volume of the modifier was added, and the solution was filled up with double distilled water, ultrasonicated and filtered.

Chromatographic conditions

The stationary phase was coated into a SPHER-100 C18 100A (250 mm x 4.6 mm, 5 μm particle size). The mobile phase was a micellar solution which was running at isocratic mode, with detection at 337 nm. The injection volume and the flow rate were 20 μL and 1 mL/min, respectively. The aliquot vials and the column remain at room temperature throughout the analysis. Composition of the optimum mobile phase was 0.15 M SDS-6% (*v/v*) pentanol-pH 7.

Chromatographic care

A special care with the chromatographic system, when dealing with micellar mobile phases, is required to obtain a maximal performance and avoid column deterioration. Following these instructions, the column has a lifespan of more than 1000 injections. A surfactant-free column must be previously saturated with the SDS monomers by flushing a micellar solution for a minimum of 2 hours at 1 mL/min. When a mobile phase is changed, the new mobile phase must be pumped for 30 min. at 1 mL/min. to rinse the column and equilibrate the stationary phase. A micellar solution should never stay motionless in the chromatographic system, to avoid the precipitation of the salts (surfactant and buffer), which would seriously damage the components of the chromatographic system, especially needle, tubes and the column. If the researcher has to work during several days with the same mobile phase, it is possible to maintain the SDS solution overnight by reducing the flow rate to a minimal value. Besides, both cases contribute to maintain a good equilibrium of the adsorbed SDS-monomers on the stationary phase. A cleaning procedure has to be implemented before switching off the pump. The SDS mobile phase must be replaced by 100 % pure water, which must be pumped at 1 mL/min during 1 hour to eliminate the sodium dihydrogen phosphate buffer. Later, the column should be rinsed with 100% methanol at 1 mL/min. flow at least for 60 min. to throw out the water and remove the adsorbed surfactant and strongly retained compounds from the stationary phase. Thereafter, the chromatographic system power is ready to be turned off. The removal process of the SDS is never complete, and the stationary phase remains modified.

Therefore, it is not recommended to use this column in MLC with other surfactants or for hydro – organic RP-HPLC.

Plasma extraction and urine sample preparation

Blood and urine sample of tuberculous patients were provided for the Clinical Biochemistry Laboratorary of the Sagar Central Hospital. The study was approved by the Ethical Comitte and conducted in compliance with the ethical principle originating in the Declaration of Helsinki. In order to obtain the plasma, blood was centrifuged at 4 500 rpm for 5 min at 4°C. After, the plasma was frozen and kept at -25ºC in amber vials. Plasma samples were thawed at room temperature before the analysis.

Plasma and urine samples were 1:5 (*v/v*) diluted with a 0.15M SDS-6% (*v/v*) pentanol-pH 7, and vigorously shaken, filtered and injected [32,33]. Samples used for the validation and quantification were prepared as the same way. For spiked samples, the appropriate amount of the adequate working solution was added before the dilution.

Results and discussion

Optimization of chromatographic conditions: pH, surfactant and modifier.

The optimization of the chromatographic conditions was performed using a working solution containing 1 μg/mL of rifampicin and rifampicin quinone. These compounds show acid/base activity, based on this fact and its structure the retention behavior definitely will depend on the pH of the mobile phase. The normal working range of a C18 column is said to be between pH 2 and 9. However, weak alkaline solutions provoke a slow and continuous hydrolysis of the C18-alkyl bond, resulting in a medium-term loss of performance of the column and a reduction of its lifespan. Therefore, the study was restricted to acid and neutral pH. As the pKa of rifampicin is

1.7, the molecule is deprotonated in all the working range of the column and for this reason pH 7 was selected for carrying out the analysis.

SDS is commonly used as an anionic surfactant which is less toxic, economic, ecofriendly and chromatographically compatible. Different concentration of SDS (0.05M-0.15M) were used for the optimization of desired mobile phase, at the fixed pH 7. When the concentration of SDS was at the minimum i.e. 0.05 M SDS, both drugs were excessively retained. The elution was enhanced when the concentration of SDS was increased consistently up to its optimum micellar working range i.e. 0.15 M. At this concentration the chromatograpic parameters were: rifampicin, 21.3, 2415 and 1.96; rifampicin quinone 27.3; 1124 and 2.87 (retention time, efficiency and asymmetry factor) respectively. As these results were considered not satisfactory, we envisage to incorporate a small amount of short chain monoalcohol, to improve the elution strenght and the peak shape. Variable concentration of organic modifier i.e. propanol (2.5-12.5%), butanol (1-7%) and pentanol (2-6%) was selected and the effect of these modifiers on the peak parameter was studied. Using propanol and butanol the chromatographic parameters were not greatly improved but still leaving room for further improvement. This promoted the use of pentanol as organic modifier which showed better results than those using of propanol and butanol.

Optimization of SDS/1-pentanol concentration

Use of an empirical strategy of testing mobile phases at several values of SDS/1-pentanol to find out the optimum mobile phase was judged to be very good. Therefore, an interpretative strategy based on a chemometric approach was applied, in order to expedite the optimization process [34]. A mathematical model, able to predict the chromatographic parameters of hydrophobic analytes from the composition of the mobile phase, was used. The Equation 1 is taken to model the retention factor:

$$K = \frac{K'_{AS} / (1+K_{AD\varphi})}{1+ [M] (K_{AM} (1+K_{MD\varphi})/ (1+K_{AD\varphi}))} \qquad \textbf{Equation 1}$$

where [M] and φ represent the concentration of SDS (M) and 1-butanol (%, v/v). The constants are partition coefficients and are described in further detail in [34]. K_{AD} and K_{AM} depend on the analyte and the surfactant, whereas K_{MD} and K_{AD} are different for each analyte, surfactant and alcohol. The simulation of the peak shape (efficiency and asymmetry) was carried out using the equation 2, which calculate the signal h(t) at each time of the chromatographic run because of the elution of the analyte:

$$h(t) = H\ exp\left[-\frac{1}{2}\left(\frac{t - t_R}{s_0 + s_1\ (t - t_R)}\right)^2\right] \qquad \textbf{Equation (2)}$$

Value h(t) indicates the signal at each time (t) of the chromatographic run because of the elution of the analyte, which is considered as an asymmetrical normal function. H depends on the concentration and sensitivity, and t_R is the retention time. The constant s_0 is the central dispersion of the Gaussian curve, whereas s_1 quantify the sleekness. These parameters remain ideally invariant for each drug and mobile phase.

The experimental values of retention factor, N and B/A of rifampicin, measured from the analysis performed using the mobile phases described in section 3.1.2 were processed by the Michrom software [31] to fit the equations 1 and 2. Thus, the theoretical values of the retention time and peak shape of behavior rifampicin can be estimated at any combination of SDS and 1-Pentanol inside the range 0.05-0.15 M, and 2-6%, respectively, by interpolation. Combining the information by different peaks which are at the same mobile phase, the theoretical values of resolution between

each pair (r_{ij}), and the global resolution (Z) were calculated using the valley-peak and denormalized product of all the r_{ij} criteria, respectively. Besides, Michrom software is able to draw simulated chromatograms, and then the analyst can easily visualize the variation of the chromatogram shape, and check the overlapping, when concentration of SDS or 1-butanol changes.

According to the chemometric model, the mobile phase providing the maximum resolution at the minimum analysis time was: 0.15 M SDS-6% 1-pentanol-phosphate buffer 0.01 M-pH 7 (R = 0.9998). The standard solution was analyzed under these conditions, and the studied rifampicin was eluted without overlapping with adequate peak shape in less than 15 min. In this mobile phase, experimental chromatographic parameters were: rifampicin (t_R: 5.8; N: 6535; B/A: 1.35) and rifampicin quinone (t_R: 11.8; N: 5238; B/A: 1.64).

Advantages of the procedure

The developed procedure shows several advantages over hydro-organic RP-HPLC, because of the use of micellar solutions. Main feature of the procedure is the simplification of the sample preparation, exploiting the possibility of direct injection allowed by MLC, eliminating need for clean-up steps. Sample is quantitatively transferred to chromatographic system, without any step with variable recovery. All these characteristics strongly reduce the global analysis time and facilitate the successive analysis of a large number of samples. Experimental procedure and the mobile phase used are biodegradable salts. The mobile phase contains only 6% pentanol as toxic and flammable organic solvent which is quite less than that normally used in hydro-organic mobile phases (which is up to 100 % and more than 40% for rifampicin using hydro-organic). Therefore, risk for the health of the laboratory staff due to the handling of toxic chemicals and waste disposal are minimized. This fits the current trend in analytical chemistry [34]. Using this analytical procedure, analyses can be performed at lower cost, because only basic chromatographic and laboratory instrumentation is used, and low amount of inexpensive reagents is required. Moreover, a large number of samples can be analyzed each day.

Method validation

The method was fully validated by following the Guideline on bioanalytical method validation issued by the European Medicines Agency (EMA). Evaluated validation parameters were: selectivity, linearity, calibration curve, lower limit of quantification, precision, accuracy, carry-over, matrix effect, stability, limit of detection, and robustness [30]. Unless specified, validation was performed using drug-free plasma and urine samples from healthy volunteers spiked with the studied rifampicin.

Selectivity

Blank plasma and urine were constructed by mixing the corresponding biological fluids, respectively, obtained from healthy volunteers (three males and three females) who did not taken any drug. These blanks were analyzed by the developed procedure. In all cases, a broad band elutes from the dead time to nearly 4.0 min (Figure 2A for blank plasma and Figure 2B for blank urine). This band corresponds to the proteins and other endogenous compounds. These compounds strongly interact with the micelles of the mobile phase, and then are barely retained on the stationary phase. No other peaks were detected beyond 3.5 min. This result is similar to that obtained in other works which were devoted to the analysis of plasma samples in MLC [35]. As the analytes show retention times > 4.5 min, there are no potentially interfering compounds, thus the procedure is selective enough to unambiguously detect the analyte. Both blanks were fortified at 1 μg/mL of rifampicin and rifampicin quinone and analyzed. Both compounds were eluted without interference at the same retention time as determined in standard solution. No other compounds were found close to those corresponding to the analyte (Figure 2C for spiked plasma and Figure 2D for spiked urine).

Calibration range and sensitivity

Nine plasma and urine spiked samples containing increasing concentrations of rifampicin and rifampicin quinone, in the range 0.05 to 50 μg/mL), were analyzed by triplicate. Parameters of the calibration curve and degree of linearity were obtained using least-square linear regression, by plotting the average AUC v.s. the corresponding concentration for both compounds. Five calibration curves were constructed in three different days (using different spiked samples) over a 1-month period, to consider influence of the between-run variability. The slope, y-intercept and determination coefficient were: (63024.5; 58402.2); (6.1; -4.2) and (0.9998; 0.9995). The procedure was found linear over the studied range.

Upper limit of quantification was set at 5 μg/mL, whereas lower limit of quantification (LLOQ) was the lowest concentration which can be measured with enough accuracy and sensitivity (see section 3.2.3), 0.05 μg/mL, for both drugs. Limit of detection was calculated following the 3.3s criterion. Standard deviation of the blank was that of the y-intercept (0.03 μg/mL, for both compounds). The lower limit of quantification was below the lower limit of the therapeutic range of rifampicin.

Accuracy and precision

These parameters were within-run determined at three concentrations (0.25, 0.50 and 1.5 μg/mL) by six replicates, in the same day. Accuracy was the quotient of the average of the found concentrations minus the true value, divided by the true value, whereas the precision was taken as the relative standard deviation of the measured peak areas.

To calculate between-run values, the same experiments were performed three days over a 1-month period, by renewing the analyzed spiked samples. Accuracy was determined as difference of the average of average values of concentrations found each day and the true value, divided by the true value. Precision was the RSD of the average values of peak area taken each day.

Values of accuracy (-3.4 to +4.0%) and precision (RSD <9.2%) were inside maximum values accepted by the guide for both parameters (less than 20% for LLOQ and less than 15% for higher values). Therefore, the method provides reliable quantitative values for rifampicin and its derivative with a low uncertainty.

Carryover effect

The carryover effect was evaluated by analyzing a plasma and urine sample spiked with rifampicin and rifampicin quinone at 5 μg/mL, and, in the following injection, the corresponding blank. No other peaks, was observed in the chromatogram near the retention time of the analytes for both biological fluids. Thus, the cross-contamination was considered negligible at concentrations within the calibration range.

Matrix effects

Possible interference of the matrix compounds was evaluated by analysis of plasma and urine samples spiked at same concentrations as for accuracy/precision, and standard working solutions (in micellar media) containing the same concentrations divided by five (to consider the dilution factor in the experimental procedure). The peak area proved to be very similar, regardless the starting environment. Therefore, the matrix effect was not significant.

Robustness

In a realistic situation, the experimental parameters randomly oscillate in a short range, which introduce a new source of variance in the instrumental response. To verify the extent of this fact, the change in the chromatographic response for rifampicin and rifampicin quinone (retention time, efficiency, asymmetry, and peak area) was studied after small changes, which can occur during the normal handling of the instrumentation: SDS concentration, 0.14-0.16 M; 1-pentanol, 5.5-6.5%; pH, 7.5-6.5; flow-rate, 0.9-1.1.

Influence of each parameter was separately studied by analyzing the standard solution using three mobile phases at: the optimal value, and the lower and upper values of the tested range, remaining the others constant. RSD of the three measurements was calculated for retention time and peak area. Results are showed in Table 1. The method is quite robust, as low variations were observed for retention time and peak area (less than 5%), within the considered range.

Table 1. Robustness Evaluation of the Developed MLC Method (Variation in RSD, %).

	Chromatographic response	Flow-rate (mL/min)	pH	1-pentanol (%, v/v)	SDS (M)
Rifampicin	t_R (min)	0.06	0.08	4.6	4.6
	N	2.2	3.8	1.9	1.7
	B/A	1.2	1.6	2.3	2.0
	AUC	3.2	2.9	2.8	2.1
Rifampicin quinone	t_R (min)	0.3	0.2	5.2	4.8
	N	3.4	2.9	1.7	2.0
	B/A	1.8	1.5	2.5	2.9
	AUC	4.0	3.4	3.2	3.5

Stability and decomposition studies

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, *etc*. 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. As reported in the literature, rifampicin is a highly unstable drug, and readily decomposes in rifampicin quinone due to oxidation [3].

The stability was studied from solutions or spiked samples containing 1.0 μg/mL of rifampicin and without rifampicin quinone. The results were found similar for plasma and urine. The investigated conditions were:

- Standard solution in a mobile phase kept in the fridge for one week (Figure 3A). Under these conditions, rifampicin was found stable up to four day (90% decay). Therefore, the standard solutions must be renewed after this period.
- Short term stability of the analyte in the processed biological fluid at room temperature for one day (Figure 3B). The analyte decays in nearly one day, and then a prepared sample must be analyzed a maximum of one day after the dilution. This is a quite long period and to not hinder the normal work at the laboratory in routine conditions. The sample throughput is limited to 96 samples per day, a high value.
- In the biological fluid long term freeze at -20ºC for two weeks (the usual storage conditions in a Hospital). A short decay (90%) was noticed at the end of the experiment. Therefore, the extracted sample can be stored until fifteen days before analysis, without affecting the measured concentration of rifampicin.

An additional conclusion of the study was that the decomposition is accelerated as the temperature increased.

Analysis of incurred samples

The developed procedure was applied to the analysis of the free plasmatic fraction of rifampicin in samples extracted from tuberculosis disease patients. The developed method was applied for the determination of rifampicin in incurred samples i.e. plasma and urine of TB patient enrolled in DOTS programme Table 2. The samples were collected from DOTS treatment center generally after 8 hours of drug administration.

The chromatogram of incurred samples of plasma and urine of TB patients are shown in Figure 4A and 4B. While observing the chromatogram at the head of chromatogram there is a broad

peak which is due to the presence of serum matrix which normally solubilized by the surfactant and elutes out in the manner as depicted at the begining of the chromatogram. As observed in the chromatogram, the developed method and obtained results shows that the analysis for the determination of rifampicin present in an incurred sample of TB patients are good in accordance with chromatogarphic parameters.

Table 2. Amount of rifampicin found in plasma and urine of 8 tubercular patients

Patient	Rifampicin in plasma µg/ml	Rifampicin in urine µg/ml
Patient 1	2	1.6
Patient 2	1.5	0.8
Patient 3	1.3	0.93
Patient 4	5	3.1
Patient 5	0.8	0.56
Patient 6	2.2	1.97
Patient 7	2.9	0.67
Patient 8	4.1	3.12

Conclusions

Micellar liquid chromatography has been demonstrated as a useful technique for the analysis of rifampicin in plasma and urine. Main advantage of this procedure is the possibility of direct injection. Therefore, plasma and urine samples were analyzed following an easy-to-handle, one-step, rapid and reproducible assay, instead of by long and tedious extractions. Hence, the main part of the experimental protocol is simplified, minimizing the probability of operator error and the use of reagents. The analytes were eluted without interference from the matrix in less than 15 min, using an isocratic mobile phase, resulting in a low global analysis time. These characteristics allow the analysis of a large number of samples per day. The method was validated by an official guideline issued by a renowned institution (European Medicines Agency) in terms of selectivity, carry-over effect, linearity, sensitivity, matrix effect, precision, accuracy, robustness and stability with adequate results, which assesses reliability of quantitative data. The procedure is eco-friendly and safe for the laboratory staff, according to the low amount of toxic chemicals handled and wasted. Moreover, it is relatively inexpensive, and then accessible to clinical laboratories with limited economic power. As there is an urgent need for therapeutic drug monitoring for TB patients to improve therapeutic rate and to reduce the side effects of the drugs. The developed method could be easily used in clinical laboratories as routine analytical tool for monitoring of DOTS patient as well as for adjusting the future dosages, according to the high sample throughput. The developed method could also be used for multi drug therapy of leprosy patient

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Conflict of interest disclosure

The authors declare that they have no financial/commercial conflicts of interest.

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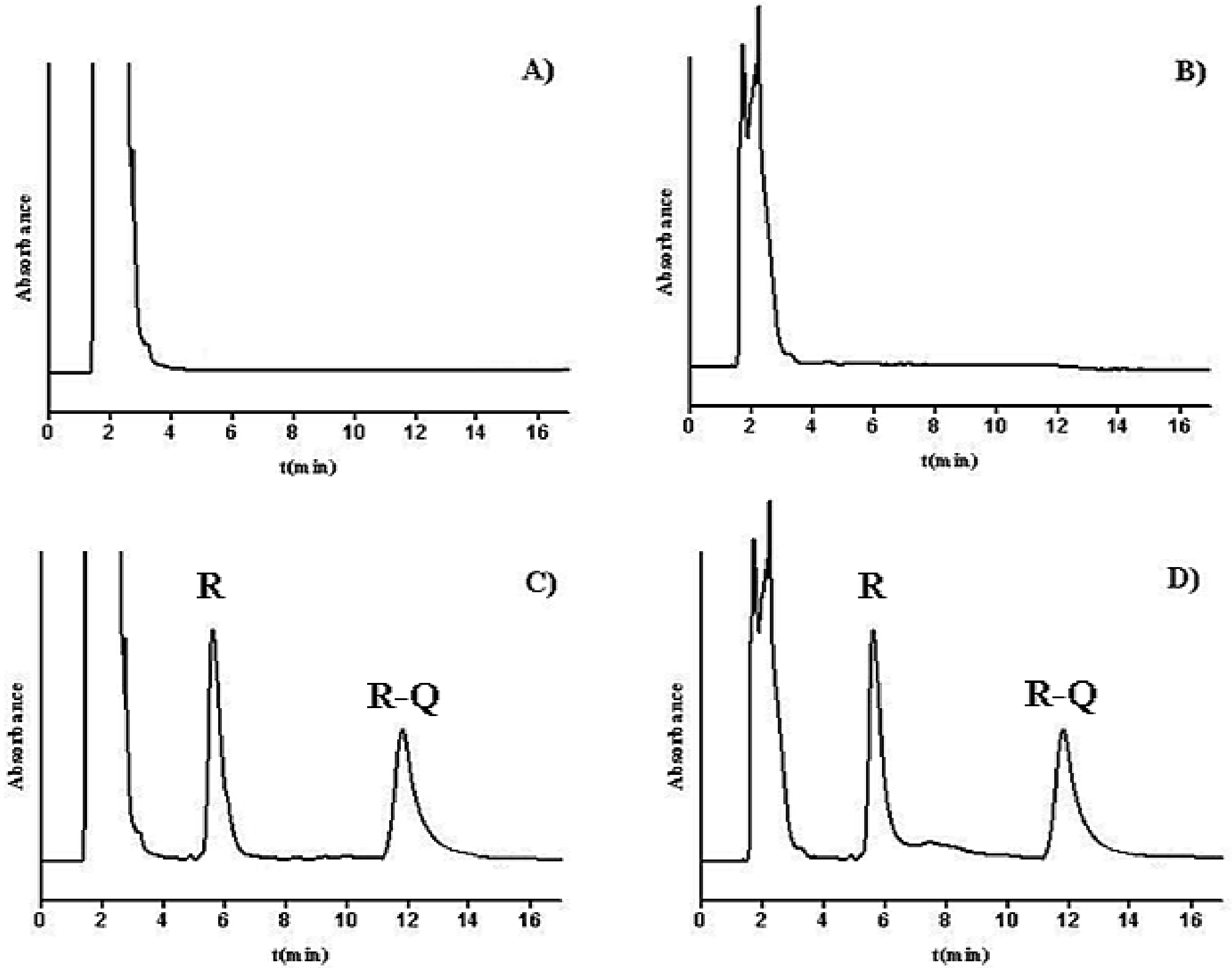
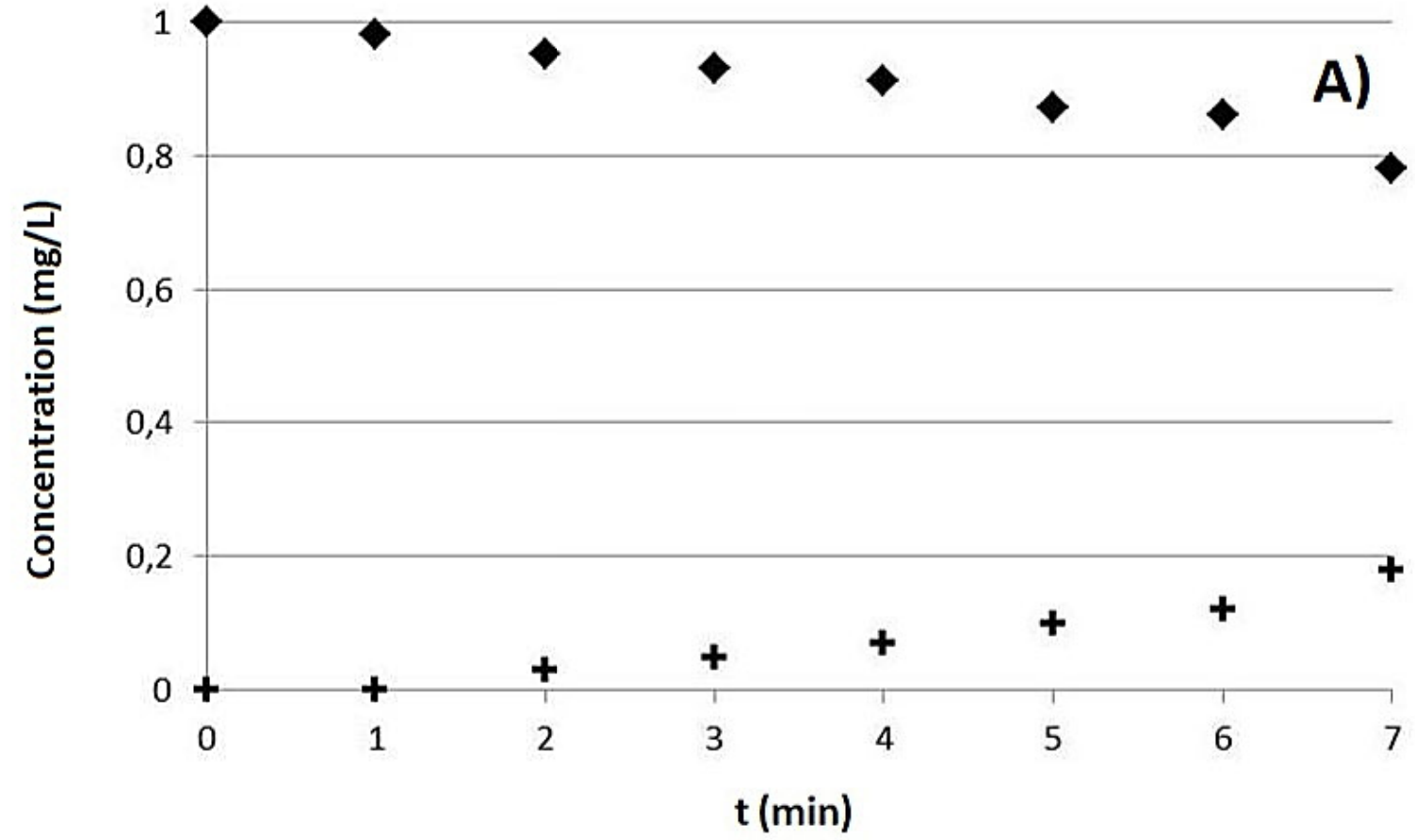
FIGURE CAPTIONS

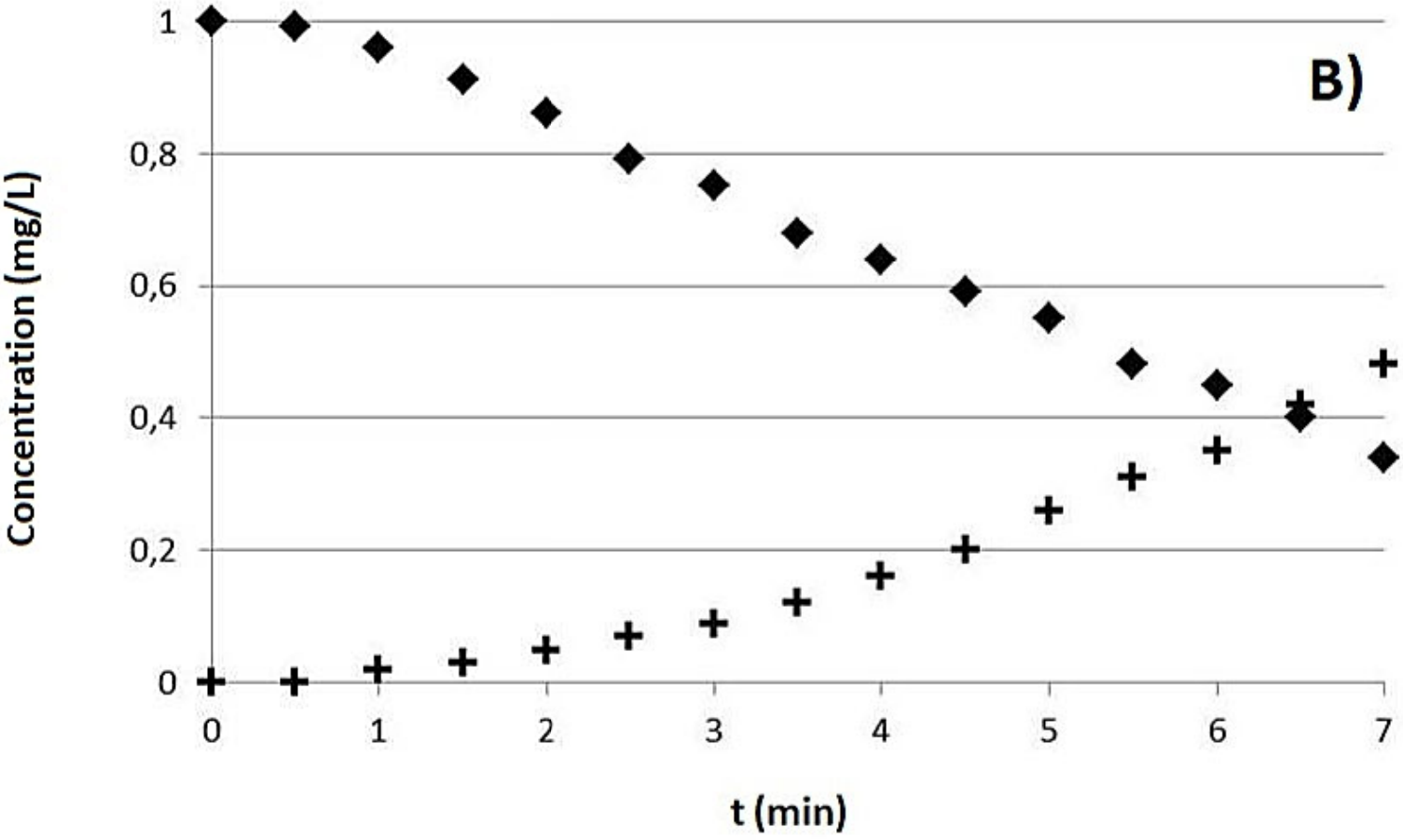
Figure 2. Chromatograms of A) blank plasma; B) urine plasma; 1-μg/mL rifampicin (R) and rifampicin quinone (R-Q) fortified C) plasma and D) urine; using the optimal conditions.

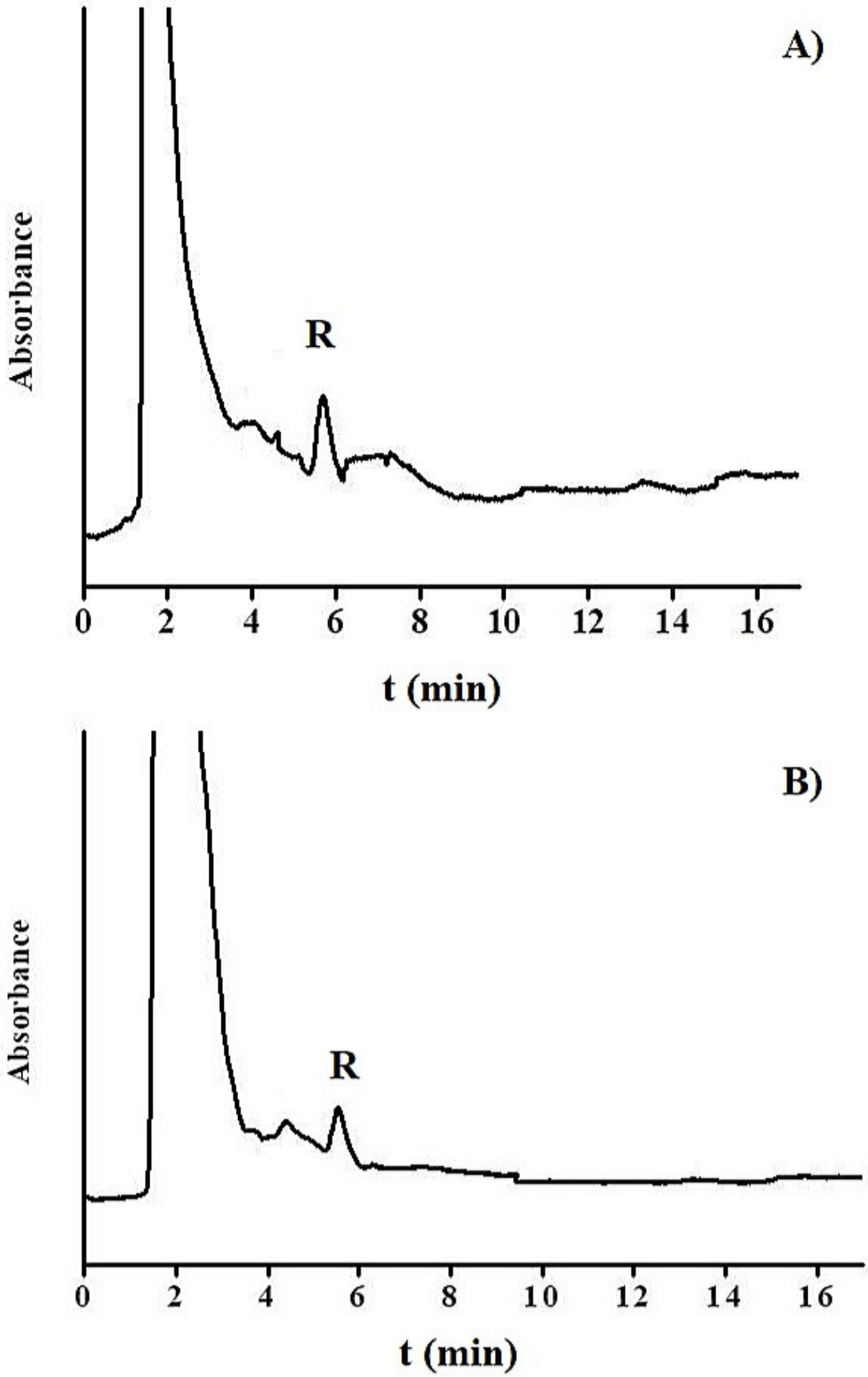
Figure 3. Kinetics of the rifampicin decomposition (♦) and rifampicin quinone formation (+) in A) standard solution kept at 4ºC and B) processed biological fluid at room temperature.

Figure 4. Chromatogram obtained from the analysis of (A) Plasma of tubercular patient (B) Urine of tubercular patient using Optimum Chromatographic Condition**s.**


A)
B)
C)
D)
R
R-Q
Absorbance
t(min)
0
2
4
6
8
10
12
14
16


A)
Concentration (mg/L)
1
0,8
0,6
0,4
0,2
0
0
1
2
3
4
5
6
7
t (min)


B)
Concentration (mg/L)
1
0,8
0,6
0,4
0,2
0
0
1
2
3
4
5
6
7
t (min)


A)
Absorbance
R
0
2
4
6
8
10
12
14
16
t (min)
B)
Absorbance
R
0
2
4
6
8
10
12
14
16
t (min)