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Rifampicin determination in plasma by stir bar-sorptive extraction and liquid chromatography

Marina Salviato Balbão[a], Carlo Bertucci[b], Mateus Machado Bergamaschi[a], Regina Helena Costa Queiroz[a,*], Wilson Roberto Malfará[a], Sônia Aparecida Carvalho Dreossi[a], Lidervan de Paula Mello[c], Maria Eugênia Costa Queiroz[c]

[a] Department of Clinical, Toxicologic and Bromatologic Analyses, Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil
[b] Department of Pharmaceutical Sciences, University of Bologna, Italy
[c] Departamento de Química, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Brazil

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ABSTRACT

A sensitive and reproducible stir bar-sorptive extraction and high performance liquid chromatography-UV detection (SBSE/HPLC-UV) method for therapeutic drug monitoring of rifampicin in plasma samples is described and compared with a liquid:liquid extraction (LLE/HPLC-UV) method. This miniaturized method can result in faster analysis, higher sample throughput, lower solvent consumption and less workload per sample while maintaining or even improving sensitivity. Important factors in the optimization of SBSE efficiency such as pH, temperature, extraction time and desorption conditions (solvents, mode magnetic stir, mode ultrasonic stir, time and number of steps) were optimized recoveries ranging from 75 to 80%. Separation was obtained using a reverse phase C_8 column with UV detection (254 nm). The mobile phase consisted of methanol:0.25 N sodium acetate buffer, pH 5.0 (58:42, v/v). The SBSE/HPLC-UV method was linear over a working range of 0.125–50.0 $\mu g\,mL^{-1}$. The intra-assay and inter-assay precision and accuracy were studied at three concentrations (1.25, 6.25 and 25.0 $\mu g\,mL^{-1}$). The intra-assay coefficients of variation (CVs) for all compounds were less than 10% and all inter-CVs were less than 10%. Limits of quantification were 0.125 $\mu g\,mL^{-1}$. Stability studies showed rifampicin was stable in plasma for 12 h after thawing; the samples were also stable for 24 h after preparation. Based on the figures of merit results, the SBSE/HPLC-UV proved to be adequate to the rifampicin analyses from therapeutic to toxic levels. This method was successfully applied to the analysis of real samples and was as effective as the LLE/HPLC-UV method.

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1. Introduction

Rifampicin (RIF) is categorized as one of the first line antituberculous agents. The ability to kill *Mycobacterium tuberculosis* is related to the concentration of drug to which the bacterium is exposed. Incomplete treatment of tuberculosis (TB), is common and the development of drug resistance [1] may usually be attributed to non-compliance with the therapeutic regime or an interrupted supply of drugs. Therapeutic drug monitoring (TDM) [2] may provide a means of determining compliance, particularly in remote areas of developing countries. Currently, plasma levels of RIF (Fig. 1) are not monitored routinely in TB patients but it is clear that this would be advantageous if a simple and effective quantitative test was available. A number of HPLC-based assays for rifampicin have been described [2–14]. Analytical methods generally require an extraction and enrichment before an analyst can perform the chromatographic separation and detection of organic compounds in aqueous matrices. Solid-phase microextraction (SPME) was successfully applied to analyze drugs in biological fluids by chromatography techniques. The principles and applications of sorptive extraction for sample preparation have been reviewed by Kawaguchi et al. [15], David and Sandra [16] and Lanças et al. [17]. A glass stir bar is coated with a potentially thick bonded absorbent layer (polydimethylsiloxane, PDMS) to give a large surface area of stationary phase, leading to a higher phase ratio and hence a better recovery and sample capacity. Transfer of the analyte from the bar is achieved either by elution with a LC solvent or GC thermal desorption. Those techniques include SPME and stir bar-sorptive extraction (SBSE). Sorptive extraction has proven to be an interesting and environmentally friendly alternative to liquid extraction. In sorptive extraction, the analytes are extracted

* Corresponding author at: Departamento de Análises Clínicas, Toxicológicas and Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café s/n, Monte Alegre, CEP 14040-903 Ribeirão Preto, São Paulo, Brazil.
E-mail address: rqueiroz@fcfrp.usp.br (R.H.C. Queiroz).

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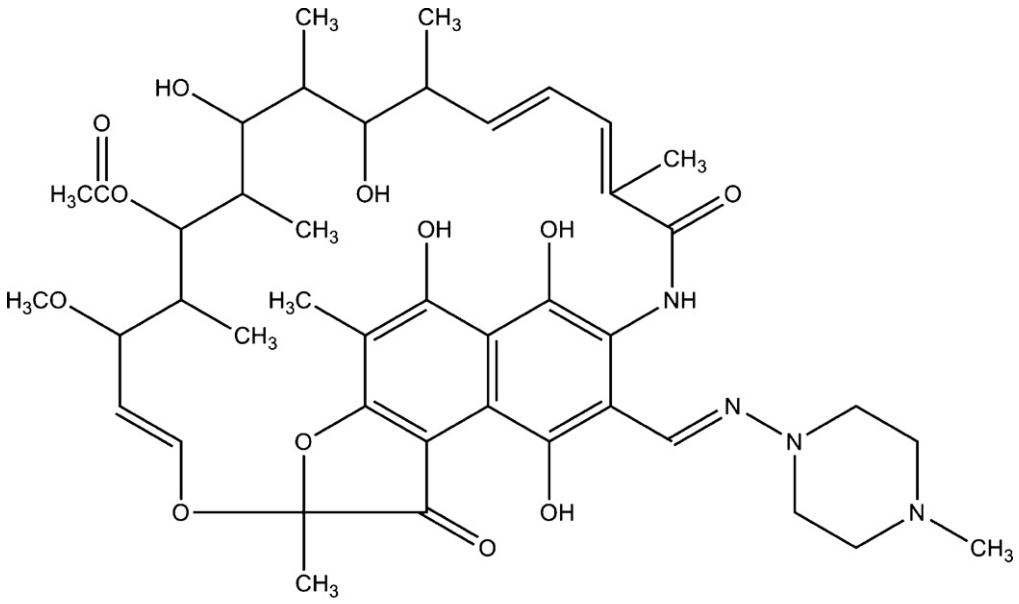

Fig. 1. Chemical structure of the rifampicin and sulindac (IS).

from the matrix (mostly aqueous) into a non-miscible liquid phase. In contrast to extraction with adsorbents in which the analytes are bound to the active sites on a surface, not only the surface area but also the total amount of the extraction phase is important in sorptive extraction. The main difference between SPME and SBSE is the much larger volume of PDMS used in SBSE, which results in higher recoveries and higher sample capacity. Kawaguchi et al. [15] also reported that the major advantage of SBSE is the higher concentration factors that can be achieved when theoretical recovery reaches 100% for solutes with $K_{O/W}$ values lower than 500 ($\log P$ greater than 2.7). RIF theoretical recoveries can be calculated for a given sample volume, selected stir bar dimensions, and KowWin around 4.8.

Recently, various methods involving SBSE were developed in order to further facilitate analysis and improve sensitivity. Novel methods that involve SBSE with in situ derivatization, SBSE with in situ de-conjugation, thermal desorption (TD) in the multi-shot mode and TD with in tube derivatization method. Those methods were applied successfully to biological samples [18–30]. The analytical methods described in the literature to analyze RIF in biological fluids usually adopt conventional sample pre-treatment techniques that are laborious, time-consuming and require large amounts of organic solvents.

The purpose of the present report is to quantify plasma RIF concentrations in tuberculosis patients using SBSE and compared with a conventional sample pre-treatment technique based on liquid:liquid extraction (LLE), also developed and validated in our laboratory, followed by HPLC-UV.

2. Experimental

2.1. Standards and chemicals

RIF was purchased from Sigma–Aldrich Inc., St. Louis, USA and sulindac (Fig. 1), the internal standard (IS) from Aldrich Chemical Company, Inc., USA. HPLC grade methanol was obtained from J.T. Baker (Phillipsburg, USA), acetonitrile was HPLC grade and was purchased from Merck (Darmstadt, Germany). Ascorbic acid and reagents used for drug extraction were analytical grade and were purchased from Merck (Darmstadt, Germany). The water used was deionised and filtered with a Milli-Q water processing system (Millipore, São Paulo, Brazil). Acetic

acid and sodium acetate were obtained from Merck (Damstadt, Germany).

2.2. Calibration curve

RIF stock solution (0.5 mg mL^{-1}) was prepared in methanol containing 0.5 mg mL^{-1} of ascorbic acid to prevent oxidation of RIF. Sulindac was dissolved in methanol at a concentration of 50 μg mL^{-1}. All stock solutions were stored at −20 °C, under dark conditions.

Routine daily calibration curves were prepared by the addition of 25 μL of a standard solution at concentrations of 0.004, 0.01, 0.02, 0.1, 0.2, 0.3, and 0.4 mg mL^{-1} of methanol to 1 mL of blank plasma (plasma from a patient not exposed to any drug for at least 2 months) aliquots, resulting in plasma drug concentrations of 0.5, 1.25, 2.5, 12.5, 25, 37.5, and 50 μg mL^{-1}. Each spiked plasma sample was processed as described in sample preparation.

2.3. SBSE accessories

The commercial stir bar Twister for sorptive extraction was obtained from Gerstel (Gerstel GmbH, Mulheim Ruhr, Germany). It consists of a 10 mm long glass-encapsulated magnetic stir bar, externally coated with 22 μg of PDMS. This layer is 0.5 mm thick, corresponding to a volume of 24 μL of PDMS. Prior to first use, the stir bars were placed in a vial containing acetonitrile and conditioned for 24 h. Among the successive extractions, the used stir bars were cleaned in methanol for 30 min at 50 °C, under magnetic stirring rate of 1200 rpm, followed by a drying step using a lint-free tissue.

2.4. Instrumentation and analytical conditions

The analysis was performed on an HPLC system consisting of a Shimadzu Model (Japan) LC 10 AD pump, a Shimadzu Model SPD 10. An ultraviolet detector, a chromatopac C-R6A integrator (Shimadzu, Japan). Chromatographic separation was achieved at room temperature on a LiChrocart® 100 RP-8 column (125 mm × 4 mm), 5 μm particle size (Merck, Damstadt, Germany). The mobile phase consisted of methanol:sodium acetate buffer 0.25 mol L^{-1}, pH 5.0 (58: 42, v/v). Flow-rate was 0.8 mL min^{-1}. The ultraviolet detector was set at 254 nm.

2.5. SBSE optimization

The influence of the pH matrix on RIF extractions was the first step evaluated, investigating different pH values from 3.0 to 9.0 (sodium acetate buffer 0.25 mol L^{-1}). In a glass vial (5 mL) sealed with a silicone septum, 25 μL of IS and 4 mL of 0.25 mol L^{-1} sodium acetate buffer were added to 200 μL of plasma sample spiked with the standard solution. The stir bar was then immersed in the sample, and the extraction was performed at a magnetic stirring rate of 1200 rpm for 15, 30, 40, 50 and 60 min.

To determine the best desorption conditions: solvents (acetonitrile, dichloromethane and mobile phase), modes (magnetic stir, ultrasonic), desorption time (15, 20, 30 and 60 min), number of desorption steps and the control of the carryover were all individually evaluated. For the desorption, the stir bars were removed with clean tweezers, rinsed lightly with Milli-Q water (1.0 mL), dried with lint-free tissue, and placed in a glass vial containing 1.0 mL of solvent, ensuring total immersion. Desorption was performed by ultrasonic treatment for 15 min at room temperature (24 °C) or by magnetic agitation for the same period at the same temperature. After the desorption process, the stir bars were removed by means of a magnetic rod and the solvent was evaporated until dryness. The dry residue was re-dissolved in 100 μL of the mobile phase and 50 μL of hexane. After shaking for 10 s in a vortex-type shaker, 20 μL of the mobile phase was injected to HPLC-UV system.

2.6. Sample preparation for LLE

The extraction consisted of the addition of 25 μL of IS, 2 mL dichloromethane to 200 μL of plasma. After shaking in a vortex-type shaker for 1 min and centrifugation at 1800 × *g* for 5 min, an aliquot (1.8 mL) of the organic phase was transferred to conic tubes and evaporated under constant air flow at room temperature. The dry residue was re-dissolved in 100 μL of the mobile phase and 50 μL of hexane. After shaking for 10 s in a vortex-type shaker, 20 μL of the mobile phase was injected into the HPLC-UV system.

2.7. HPLC-UV method validation

Drug recovery was determined at three different concentrations in blank plasma. Plasma samples with the drugs were extracted in triplicate according to the procedure proposed. The recoveries were calculated by comparing the UV-peak areas of the spiked samples with the direct injection of standard solutions of equal concentrations.

To determine the intra-assay precision, aliquots (n = 10) of blank plasma containing the standard solutions of the drugs at three concentrations were analysed by the method proposed. To determine the inter-assay precision, blank plasma containing the standard solutions at the same concentrations were analysed on 10 consecutive days. Linearity was obtained by analysing blank plasma samples (n = 3) containing standard solutions of drugs at concentrations of 0.01–50 μg mL^{-1}. The concentration range was estimated on the basis of the regression curve ($y = ax + b$) and correlation coefficient (r^2).

The limit of detection (LOD) was determined as the lowest concentration giving a response of three times the average of the baseline noise defined from five determinations. The limit of quantification (LOQ) was considered the lowest concentration quantified with a coefficient of variation less than 10% obtained from five determinations. The selectivity of the method was evaluated by analysing several drugs normally combined with this drug. Stability studies carried out directly on plasma at high (20 μg mL^{-1}) and low (0.2 μg mL^{-1}) concentrations of RIF indicated that samples were stable for at least 3 months when stored at −20 °C.

2.8. Sample collection

Blood samples from patients receiving RIF were collected after filling out a form containing the patient's name, sex, age, weight, prescribed medication, dose and combined medications. Blood samples from patients in steady-state plasma concentrations of RIF were obtained in the morning with heparin (Liquemine®) immediately before drug administration. After centrifugation, plasma samples with ascorbic acid (1 mg mL^{-1}), were stored at −20 °C until analysis.

Pooled blank plasma samples used for development and validation of the procedure were obtained from a local blood bank. The principles embodied in the Helsinki Declaration were adhered to, and the Ethics Committee at the University of São Paulo in Ribeirão Preto, Brazil approved the study.

3. Results and discussion

The SBSE variables, such as time, temperature, pH matrix, ionic strength and desorption conditions were optimized to reach drug partition equilibrium in a shorter analysis time, and to obtain adequate sensitivity to work in the therapeutic interval. The

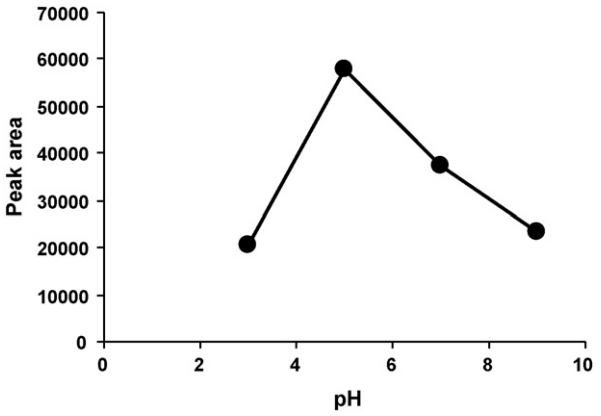

Fig. 2. Effect of the matrix pH on the SBSE efficiency rifampicin in plasma sample.

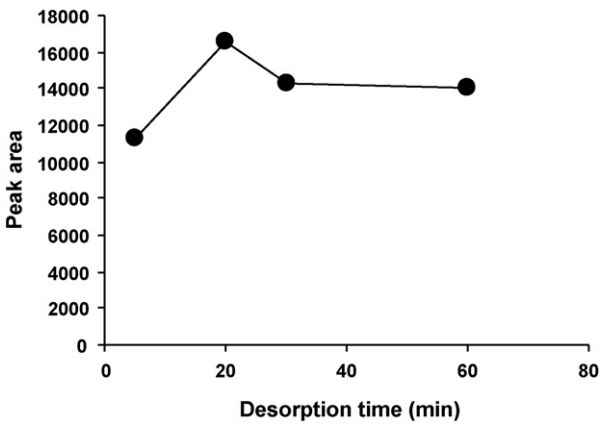

Fig. 4. SBSE desorption time profile of rifampicin in plasma sample.

sample volume, stirring speed and stir bar dimensions were maintained constant throughout optimization. The sensitivity of the SBSE/HPLC-UV method was improved by diluting the samples with the 0.25 mol L^{-1} sodium acetate buffer solution to pH 5.0. RIF is a zwitterions compound with an acidic pK of 1.7 (4-hydroxy) and a basic pK of 7.9 (3-piperazine). Consequenty, RIF, related to 3-piperazine, was partially in the non-ionic form in this solution enabling samples to be extracted by the PDMS phase (Fig. 2). The sample dilution favours the SBSE process. The addition of NaCl did not alter the efficiency of the SBSE process. Fig. 3 shows representative time extraction profiles (15–60 min) at ambient temperature (24 °C). A temperature increase from 38 to 50 °C resulted in a decreased amount of the extracted drug. Degradation was observed especially at the lowest concentration of RIF (0.5 μg mL^{-1}). RIF concentrations at 38–50 °C were 76.6 and 56.8% those of the drug extracted at 24 °C, respectively. The impact of heat-inactivation on RIF stability was also determined following the experimental conditions (40 min, 58 °C) adopted by of Hartkoorn et al. [13], obtaining similar resuts.

The desorption conditions were tested to ensure effective removal of the extracted analytes from the SBSE device. Acetonitrile yielded the best results among the desorption solvents investigated (dichoromethane and mobile phase). The recovery obtained by dichloromethane and mobile phase were respectively 50% and 65% relative to the recovery obtained by acetonitrile.

The peak areas increased from 5 to 20 min, but remained nearly constant for desorption times of 20–60 min corresponding to the complete desorption of drugs (Fig. 4).

Based upon these data, we concluded that the best SBSE experimental conditions among those investigated for RIF assay (Figs. 2–4) were the following: 200 μL of plasma sample modified with 4 mL acetate buffer (pH 5.0), extraction under magnetic stirring for 50 min, followed by off-line liquid drug desorption by immersion of the PDMS bar in acetonitrile under magnetic stirring for 20 min.

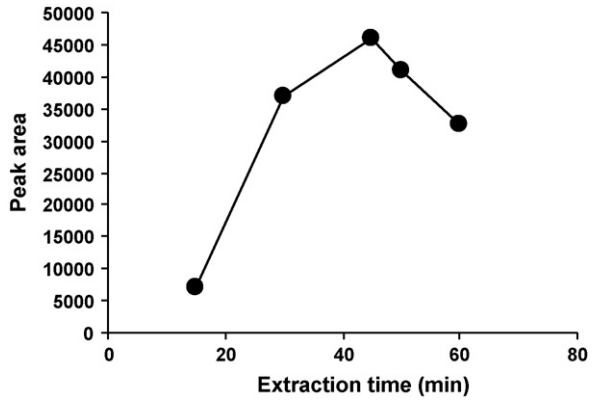

Fig. 3. SBSE time extraction profiles of rifampicin in plasma sample.

Ascorbic acid was necessary in the standard solutions and in plasma samples to prevent oxidation of RIF. In addition, all the studies had to be performed away from direct light as RIF is known to be light sensitive. Methanol calibration solutions and samples plasma were stored for 3 months at −20 °C and protected from any light source. Short-term stability studies in plasma samples failed to disclose any degradation in samples thawed and prepared at $t = 0$, 3, 6, 9, and 12 h. The response for RIF in the samples prepared and analysed after 12 h were 92.7 and 95.4% of the response at $t = 0$ h at RIF plasma concentrations of 0.2 and 20 μg mL^{-1} respectively. Allanson et al. [2] observed a degraded response at lower concentration (0.5 μg mL^{-1}) in the freeze–thaw samples. Hartkoorn et al. [13] showed there was a small decrease in the measured drug concentration for all QC plasma samples, a difference only significant for the lowest concentration (0.15 μg mL^{-1}). However, they observed a highly significant rifampicin loss when peripheral blood mononuclear cells were used, with a 51% loss of RIF during isolation (by density gradient centrifugation and washing steps). Kawaguchi et al. [15] also reported a 40% loss of RIF after 48 h in standard solutions of 1 mg mL^{-1} at room temperature. Their study also showed that the standards were reliable only if the analysis was carried out within 8–12 h after preparation.

The selectivity of the developed method is demonstrated by representative chromatograms from a drug-free human plasma sample, and the same sample spiked with RIF at therapeutic interval concentrations (Fig. 5A and B), which showed the ability of the method to measure the drug unequivocally in the presence of endogenous plasma components. Additional drug-free human plasma samples from several individuals were tested and showed no significant interference at the retention times of the analytes.

RIF may be prescribed in combination with different antibiotic agents and other drugs, so it was important to assess probable interferences from potentially co-administered compounds. No interference was observed after the extraction procedures (SBSE and LLE) of the drugs normally associated with RIF in patients with tuberculosis: isoniazid, pyrazinamide, ethambutol, sulbactam, minocyclin, ofloxacin, ciprofloxacin, norfloxacin, claritromicin, dapsone, monoacetyldapsone, clofazimine, cefalexin, diazepam and diclophenac. The same was true for dexamethazone, hydrochlorothiazide, methoclopramide, acetaminophen, caffeine, salicylic acid, sulphamethazaxol, metoprolol, propanolol, amiodarone, cimetidine, ranitidine and prednisone.

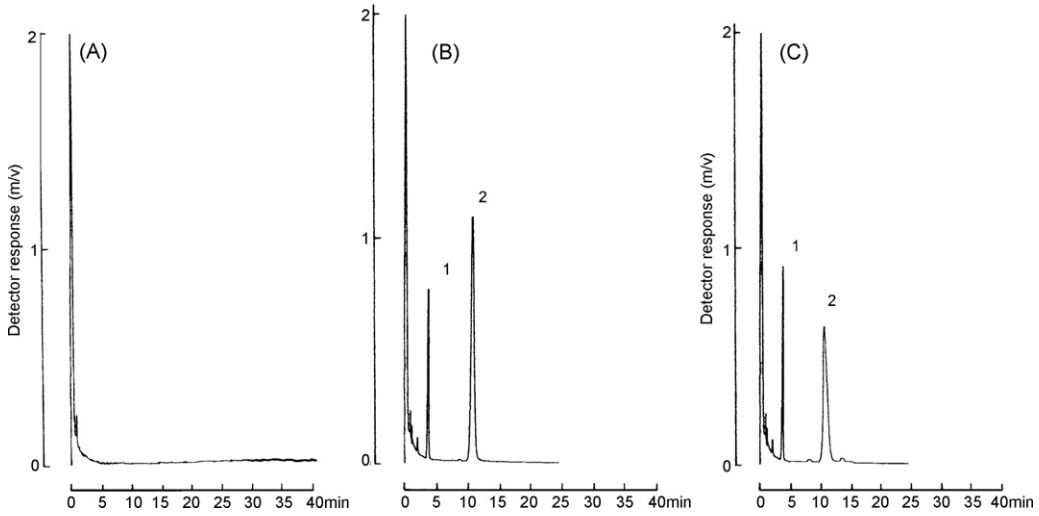

Fig. 5. (A) SBSE-HPLC-UV chromatograms obtained from a blank plasma; (B) blank plasma spiked with (1) 50 μg mL^{-1} of IS, sulindac and (2) 3.45 μg mL^{-1} of rifampicin; (C) Clinical plasma sample of tuberculosis patient: (1) IS, sulindac and (2) rifampicin; isoniazid and ethambutol do not interfere.

Table 1
Comparison of linearity, limit of quantification and recovery of rifampicin by stir bar-sorptive extraction vs. liquid:liquid extraction followed by HPLC-UV analysis in plasma samples.

Linearity SBSE/LLE (μg mL^{-1})	Linear regression r^2	LOQ (μg mL^{-1})	Recovery SBSE vs. LLE	
	SBSE vs. LLE	SBSE vs. LLE	Concentration (μg mL^{-1})	Results (%) (n = 5)
Rifampicin				
(0.125–50) vs. (0.125–50)	$y = 0.0626x - 0.0425$ 0.9951	0.125/0.05	50	78.3 vs. 91.1
			12.5	75.8 vs. 90.0
	$y = 0.02997x + 0.03189$ 0.998		0.5	76.1 vs. 88.3

LLE: liquid:liquid extraction; vs.: versus; SBSE: stir bar-sorptive extraction; LOQ: limit of quantification.

The average recovery and linearity of the SBSE and LLE/HPLC methods were determined with plasma samples spiked with analytical standards resulting in concentrations ranging from 0.125 to 50.0 μg mL^{-1}. The regression equations and corresponding correlation coefficients for RIF are listed in Table 1. The accuracy and intra- and inter-day precision were assessed by replicate analysis of plasma samples spiked with standards at three different concentrations (Table 2).

This study demonstrated that the coupling of SBSE with HPLC-UV is an appropriate technique for RIF determination in plasma samples. The developed method has many practical advantages over other methods described in the literature, including: simplicity of the extraction method, small sample volume (200 μL), solvent-free, selectivity and high stability. The method using liquid desorption showed linearity over the range of 0.125–50 μg mL^{-1}, precision (R.S.D. < 10%), and LOD and LOQ of 0.09 and 0.125 μg mL^{-1}.

Table 2
Comparison of intra- and inter-day precision and accuracy of the rifampicin by stir bar-sorptive extraction vs. liquid:liquid extraction followed by HPLC-UV analysis in plasma samples.

Drugs (μg mL^{-1})	Precision intra-assay CV (%) n = 10 SBSE vs. LLE	Precision inter-assay CV (%) n = 5 SBSE vs. LLE	Accuracy Error (%) SBSE vs. LLE
Ryfampicin			
25	3.6 vs. 3.5	5.4 vs. 7.4	3.4 vs. 4.2
6.25	8.7 vs. 6.5	3.9 vs. 5.6	5.0 vs. 3.5
1.25	7.3 vs. 7.8	3.5 vs. 5.0	6.2 vs. 7.2

CV: coeficient of variation; LLE: liquid:liquid extraction; SBSE: stir bar-sorptive extraction.

3.1. Clinical application of the method

Fig. 5A and B shows that plasma components did not interfere with RIF and internal standard. Isoniazid, ethambutol and pyrazinamide are often co-administered with RIF as antituberculosis drugs. Fig. 5C shows a chromatogram of a plasma extract from a tuberculosis patient treated chronically with 450 mg of RIF, 300 mg of isoniazid and 1.0 g of ethambutol, in which RIF was estimated to be 1.28 μg mL^{-1}. Analysis of plasma samples treated with these drugs showed that these drugs did not interfere.

4. Conclusion

The validated SBSE/HPLC-UV method presents high sensitivity and enough stability to allow the quantification of RIF in human plasma. The SBSE technique, characterized by small sample volumes and a simple work-up procedure, was successfully applied to the analysis of real samples demonstrating its suitability for therapeutic drug monitoring applications.

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