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Short communication

Automated determination of rifampicin in plasma samples by in-tube solid-phase microextraction coupled with liquid chromatography

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ABSTRACT

A sensitive and automated method is described for determination of rifampicin in plasma samples for therapeutic drug monitoring by in-tube solid-phase microextraction coupled with liquid chromatography (in-tube SPME/LC). Important factors in the optimization of in-tube SPME are discussed, such as coating type, sample pH, sample draw/eject volume, number of draw/eject cycles, and draw/eject flow rate. Analyte pre-concentrated in the polyethylene glycol phase was directly transferred to the liquid chromatographic column by percolation of the mobile phase, without carryover. The method was linear over the 0.1–100 μg/mL range, with a linear coefficient value (r^2) of 0.998. The inter-assay precision presented coefficient of variation ≤1.7%. The effectiveness and practicability of the proposed method are proven by analysis of plasma samples from ageing patients undergoing therapy with rifampicin.

1. Introduction

Tuberculosis remains a major health public concern and is the single most deadly infectious disease [1,2]. Rifampicin (Fig. 1), a semi-synthetic macrocyclic complex with antibiotic effect derived from *Streptomyces mediterranei* is a member of the rifamycin class of antibiotics used for treatment of tuberculosis and other infectious diseases. Rifampicin is categorized as one of the first-line antituberculous agents. The ability of a drug to kill *Mycobacterium tuberculosis* is also related to the drug concentration to which the bacterium is exposed [1]. Incomplete treatment of tuberculosis is common, and the development of drug resistance may usually be attributed to non-compliance with the therapeutic regimen or interrupted drug supply [2–4].

The use of rifampicin can produce potential side-effects like hepatotoxicity, allergic rashes, appetite loss, nausea, or immunological disturbances [3]. Therapeutic drug monitoring allows determination of the best dosage and enables adaptation of the therapeutic regimen to each patient, thereby optimizing the therapeutic benefits while minimizing the risk of side effects [1,5]. Currently, rifampicin plasma levels are not routinely monitored in tuberculosis patients, but it is clear that this would be advantageous if a simple and effective quantitative test was available [1,2].

Analytical methods generally require extraction and enrichment before an analyst can perform the chromatographic separation and detection of organic compounds in aqueous matrices. The most commonly employed techniques for rifampicin extraction from biological fluids have been liquid–liquid extraction and solid phase extraction [6–11]. However, these conventional techniques involve complex or very long extraction procedures, which notably increase the analysis time and organic solvent consumption [1,6]. Modern trends in analytical chemistry are geared toward simplification, miniaturization of the sample preparation system, and minimization of organic solvent and sample volumes.

In-tube solid-phase microextraction (in-tube SPME), an effective sample preparation technique, has been successfully applied to the analysis of drugs in biological fluids [12,13]. In-tube SPME uses an open tubular fused-silica capillary column as an extraction device. Organic compounds in aqueous samples are extracted and concentrated into the stationary phase of the capillary column by repeated draw/eject cycles of the sample solution, and they can be directly transferred to the liquid chromatographic column [12].

In-tube SPME is an ideal sample preparation technique because it is fast to operate, easy to automate, solvent-free, and inexpensive. Moreover, on-line in-tube SPME allows for continuous extraction, concentration, desorption, and injection using an autosampler, which is usually employed in combination with high performance liquid chromatography (LC) and liquid chromatography–mass spectrometry [12,13].

In this work, an in-tube SPME/LC method was developed and validated for rifampicin determination in plasma samples for therapeutic drug monitoring. The applicability of the in-tube SPME/LC

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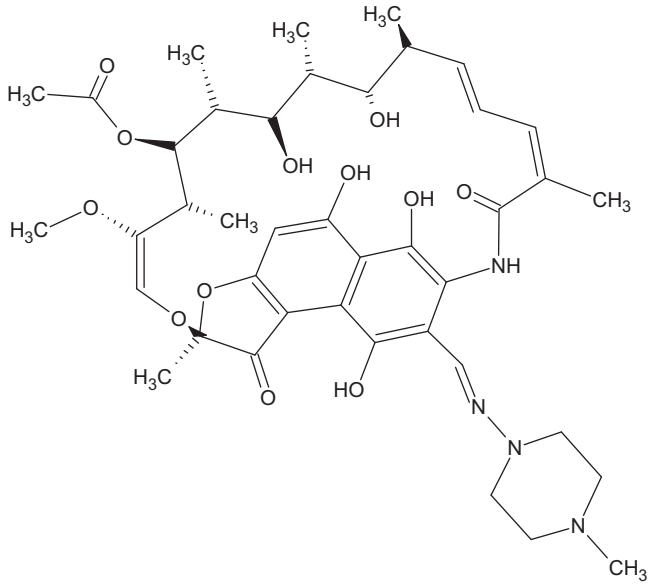

Fig. 1. Rifampicin chemical structure.

method is demonstrated by analysis of plasma samples from ageing patients undergoing therapy with rifampicin.

2. Experimental

2.1. Reagents and analytical standards

Rifampicin was purchased from Sigma (Sigma–Aldrich, Inc. St. Louis, USA) and carbamazepine (internal standard) was kindly donated by Ciba-Geigy (São Paulo-SP, Brazil). Ascorbic acid was acquired from Merck (Darmstadt, Germany). The standard working solutions of rifampicin and the internal standard were prepared by diluting the stock solutions (1.0 mg/mL in methanol containing ascorbic acid (1.0 mg/mL), to prevent rifampicin oxidation). These rifampicin standard solutions remained stable for 3 months, at −20 °C.

Methanol, HPLC grade, was supplied by J.T. Baker (Phillipsburg, NJ, USA). The water used to prepare the mobile phase had been purified by a Milli-Q system purchased from (Millipore, São Paulo-SP, Brazil). Monobasic and dibasic phosphate and sodium acetate were obtained from Merck (Darmstadt, Germany).

2.2. Instrument and analytical conditions

The in-tube SPME/LC system consisted of a pre-extraction segment and LC–UV analyses, which included a Pro Star Varian (model 230, Walnut Creek, California, USA) liquid chromatograph with a Varian autosampler. Signal was monitored by a UV detector set at 225 nm.

The chromatographic separations were performed using a LiChrospher®60 RP-select B (C18) column (5 μm, 250 mm × 4 mm, Merck), at room temperature (25 °C), with a mobile phase consisting of phosphate buffer solution (0.05 mol/L, pH 5.0)/acetonitrile 60/40 (v/v), respectively, in isocratic mode, at a flow rate of 1.0 mL/min. The mobile phase was filtered and degassed prior to use.

2.3. Sample collection

Blood samples from patients receiving rifampicin were collected after subjects had filled out a form containing the following information: name, gender, age, weight, prescribed medication, dose, and combined medications. Blood samples from patients with steady-state plasma rifampicin concentrations were collected in the morning with heparin (Liquemine®) immediately before drug administration. Pooled blank plasma samples used for development and validation of the chromatographic method was obtained from a local blood bank. The principles embodied in the Helsinki Declaration were adhered to, and the Ethics Committee of the University of São Paulo in Ribeirão Preto, Brazil, approved the study.

2.4. Preparation of plasma samples

The proteins of the plasma samples were precipitated before the in-tube SPME analysis, to prevent plugging of the capillary column. 0.5 mL of the reference blank plasma sample was spiked with a rifampicin standard solution (100 μL, 100.0 μg/mL) and internal standard (30 μL, 100.0 μg/mL). Acetonitrile was added to the plasma in a 2:1 (v/v) proportion, respectively. Then, the samples were immediately vortexed for 3 min and centrifuged at 3000 rpm for 10 min. The supernatant was collected and dried under N_2 flow. The dry extract was resuspended with 0.5 mL buffer solutions.

2.5. Optimization of the in-tube SPME process

The fused-silica capillary was fixed in the place of the injection loop LC autosampler. The capillary connections were facilitated by microtight tubing sleeves placed at each end of the capillary. Capillary columns with different stationary phases were evaluated: 14% cyanopropylphenylmethylpolysiloxane (OV1701) (80 cm × 0.25 mm I.D. and 0.05 μm thickness), polyethylene glycol (60 cm × 0.32 mm I.D. and 0.05 μm thickness), and uncoated fused silica (80 cm × 0.25 mm I.D.).

Optimization of the in-tube SPME variables was performed in a glass vial (1.5 mL, Sun Sri, USA) sealed with a screw cap containing a silicone septum. After plasma protein precipitation, 0.5 mL buffer solution was added to the dry extract. The samples were vortexed for 10 s before extraction. The vials were then placed in the autosampler, for optimization of the following in-tube SPME variables: sample solution draw/eject volume, from 50 μL (volume slightly larger than that of the capillary, 39–48 μL) to 250 μL (column capacity and the capacity from the injection needle to the tip of the column); pH of the buffer solutions (3.0, 5.0, 7.0, and 9.0); draw/eject cycles (1, 5, 10, 15, 20, and 25), and draw/eject flow rate (125, 315, and 625 μL/min).

Extraction of each sample was possible by repeatedly aspirating (draw) and dispensing (eject) the sample through the capillary. Desorption of the extracted analytes was then possible by redirecting the mobile phase through the extraction capillary column, switching the six-port injection valve from the load to the inject position, in order to transport the analytes to the analytical column. After the desorption procedure, the capillary column was washed with water/methanol 50:50 (v/v).

2.6. Analytical validation

Analytical validation of the in-tube SPME/LC method was carried out using blank plasma samples spiked with rifampicin standard solutions at concentrations that included the therapeutic drug levels. Linearity was evaluated by a calibration curve constructed using linear regression of the rifampicin/internal standard peak area ratio (y) versus the rifampicin nominal plasma concentration (x, μg/mL). The calibration curve was prepared by addition of 50 μL of a standard solution, which resulted in rifampicin plasma concentrations of 0.1, 10, 25, 50, 75, and 100 μg/mL.

Accuracy and inter-assay precision values were determined by calibration curves using quintuplicate in-tube SPME/LC assays of

the blank plasma samples spiked with rifampicin, and internal standard at three levels (high, medium, and low).

Quality control samples (single bath) were prepared with blank plasma samples spiked with rifampicin standard solutions at three concentration levels, as follows: one near the LOQ, one near the centre, and one near the upper boundary of the calibration curve. These samples were separated into aliquots, frozen in appropriate containers, and applied for the analytical validation assays.

Precision around the mean value should not exceed 15% of the coefficient of variation. As for accuracy, the mean value should be within ±20% deviation of the nominal value.

Accuracy values were calculated by comparison between rifampicin concentrations added to the plasma samples with plasma drug concentrations determined by the calibration curve.

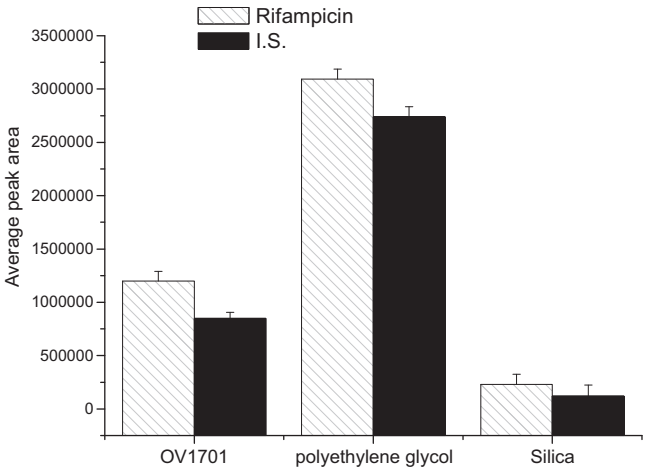

Fig. 2. Evaluation of capillary columns for rifampicin in-tube SPME.

3. Results and discussion

3.1. Optimization of the in-tube SPME variables

Optimization of the in-tube SPME variables such as sample volume, draw/eject cycles, and flow rate was carried out to shorten the time required to reach the sorption equilibrium.

The polyethylene glycol phase (liquid phase and polar) has been successfully applied for analysis of drugs in biological fluids that have a polar group [14–16]. According to Fig. 2, rifampicin presented the highest partition coefficient with polyethylene glycol. Furthermore, the polyethylene glycol phase (chemically cross-linked with the inner wall of the fused-silica capillary) was stable in the presence of the mobile phase [12].

According to Kataoka et al., an increase in the number and volume of draw/eject cycles can enhance the in-tube SPME extraction efficiency, but bandwidth may widen, and peak broadening may be observed [12]. In this work, the maximum extraction efficiency was obtained by aspirating 200 μL of the sample in each cycle (Fig. 3a). Sample volumes higher than this value did not improve the extraction efficiency.

Generally, it is possible to increase the efficiency of the analyte-extraction-to-stationary phase in SPME by changing the pH and the salt level of the sample solution [12]. Although salting out increases the extraction efficiency for fiber SPME, it should block the column due to salts deposits within in-tube SPME [12,17]. Therefore, a study about the influence of ionic strength on the SPME extraction process was not carried out.

The polyethylene glycol phase should present higher extraction efficiency for species in their non-ionized form, so the pH of the matrix was adjusted by addition of a buffer solution. The highest average peak area was obtained at pH 7 (Fig. 3b). An important property of rifampicin is its zwitterionic nature (pK_a values of 7.9 and 1.7) [4]. Consequently, rifampicin (3-piperazine) in the

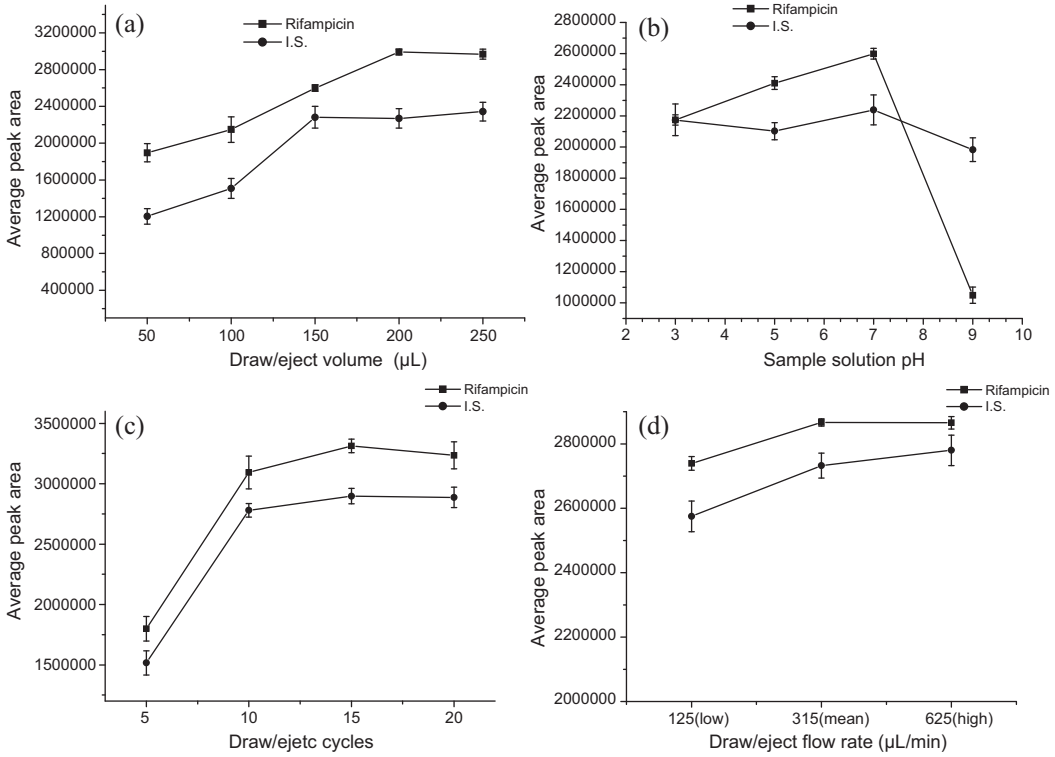

Fig. 3. Optimization of the in-tube SPME variables: (a) draw/eject volume, (b) sample pH, (c) draw/eject cycle number and, (d) draw/eject flow rate.

non-ionic form in plasma sample at pH 7 increased the extraction efficiency.

Furthermore, rifampicin is more stable in solutions at pH 7, once it can undergo hydrolysis in acidic media (pH ≤ 4.5), thereby generating 3-formyl rifampicin and 1-amino-4-methyl piperazine. In alkaline conditions (pH ≥ 7.5), rifampicin can undergo oxidation, producing rifampicin quinone [18]. Additionally, interference of the plasma proteins in the SPME process was also minimized by dilution of the sample with the phosphate buffer solution. This procedure decreased the plasma matrix viscosity and improved diffusion coefficients, considering that the efficiency of the plasma protein precipitation process was less than 100%.

Organic compounds in aqueous samples are extracted and concentrated into the stationary phase of the capillary column by repeated draw/eject cycles of the sample solution [12]. As observed in Fig. 3c, the partition equilibrium was reached with 10 draw/eject cycles of 200 μL sample, which resulted in adequate analytical sensitivity for rifampicin analysis in plasma samples. The optimal flow rate of draw/eject cycles was 315 μL/min (Fig. 3d) in our experiments.

For the in-tube SPME desorption process, online elution was performed by redirecting the mobile phase through the capillary column. The mobile phase (phosphate buffer solution (0.05 mol/L, pH 5.0)/acetonitrile 60/40 (v/v)) favored rifampicin desorption from the polyethylene glycol phase. After this procedure, no carryover was observed during blank assays.

On the basis of these data, the extraction equilibrium was reached with 10 draw/eject cycles, at 315 μL/min, for a 200 μL sample solution (pH 7).

4. Analytical validation

Based on our previous study on the short-term stability of rifampicin in spiked plasma samples, analyzed after 12 h, it was shown that high stability at concentrations ranging from 0.2 to 20 μg/mL [1]. Moreover, bench-top and autosampler stability test revealed that rifampicin in plasma samples is stable for 8 and 15 h at room temperature, respectively [2,7]. Perri et al. reported that rifampicin degradation in plasma samples in 3 months was less than 20% (long-term stability studies) [7].

Ascorbic acid was added to the standard solutions and plasma samples, to prevent rifampicin oxidation. In this condition, rifampicin (protected from any light source mode) was stable in the plasma samples both in bench top (controlled room temperature) and in storage conditions (3 months at −20 °C).

Among the compounds tested as internal standard in this study, carbamazepine was closely related to rifampicin, mainly in terms of partition coefficient with extraction phase. Moreover, carbamazepine presented adequate chromatographic resolution, and stability in the analysis conditions, and it can be added to samples at concentrations similar to those of rifampicin. The concomitant use (polytherapy) of carbamazepine and rifampicin increases the carbamazepine plasma levels that could cause toxicity symptoms. Consequently, carbamazepine is not usually co-administered with rifampicin [19].

p-Dimethylaminobenzoic acid [20] was also tested as internal standard in this work. This compound (retention time = 4.5 min) did not coelute with rifampicin and it presented partition coefficient with the polyethylene glycol phase similar to that obtained for rimfampicin. Therefore, p-dimethylaminobenzoic acid can be used as an alternative internal standard to carbamazepine.

The selectivity of the developed method was demonstrated by representative chromatograms of blank plasma samples (Fig. 4a), and blank plasma samples spiked with rifampicin (Fig. 4b). No endogenous plasma compounds co-eluted (same retention time) with rifampicin.

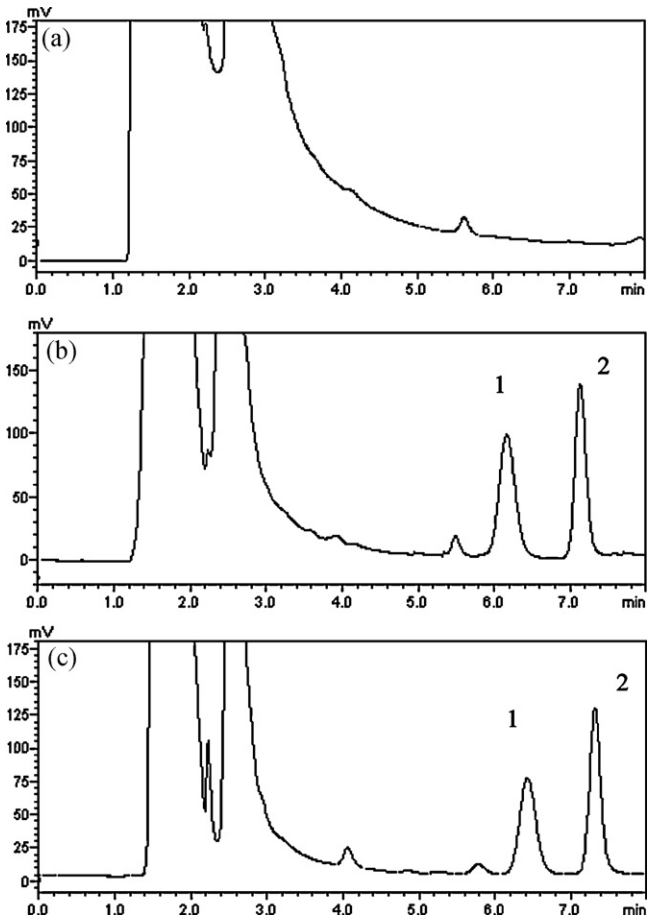

Fig. 4. Rifampicin in-tube SPME/LC chromatograms (a) blank plasma sample, (b) plasma sample spiked with rifampicin (10.0 μg/mL) (1) and I.S. (3.0 μg/mL) (2) and (c) tuberculosis patient plasma sample, determined average concentration (7.0 μg/mL) (1) rifampicin and (2) I.S.

Rifampicin may be prescribed in combination with different antibiotic agents and other drugs, so it is important to assess probable interferences from potentially co-administered compounds. The following drugs were thus evaluated: isoniazid, pyrazinamide, ethambutol, sulbactam, minocycline, ofloxacin, ciprofloxacin, norfloxacin, clarithromycin, dapsone, monoacetyldapsone, clofazimine, cefalexin, diazepam, diclofenac, dexamethasone, hydrochlorothiazide, metoclopramide, acetaminophen, caffeine, salicylic acid, sulfamethozazole, metoprolol, propanolol, amiodarone, cimetidine, ranitidine, and prednisone. On the basis of the retention times, it was verified that these drugs did not co-elute with rifampicin or the internal standard.

The linearity of the in-tube SPME/LC method was determined by means of plasma samples spiked with rifampicin analytical standards, resulting in a linear concentration interval ranging from the 0.1 up to 100 μg/mL. The obtained regression equation and the

Table 1
Inter-assay precision and accuracy of in-tube SPME/LC method.

Added concentration (μg/mL)	Determined concentration $\bar{x} \pm s$	Inter-assay precision ($n = 5$) CV %	Accuracy % ($n = 5$)
10.0	8.6 ± 0.099	1.2	86
25.0	20.9 ± 0.25	1.2	83
75.0	69.3 ± 1.17	1.7	92

Table 2
Comparison between the in-tube SPME/LC method and conventional methods described in the literature for rifampicin analysis in plasma samples.

Extraction technique	Detection	Linearity range (μg/mL)	LOQ (μg/mL)	Inter-assay precision (CV%)	Sample volume (μL)	Ref.
SPE[a]	LC/UV	0.5–20	0.5	≤7.2	450	[1]
LLE[b]	LC/UV	0.125–50	0.125	<10.0	200	[2]
LLE	LC/UV	1–50	0.05	<15.0	100	[6]
LLE	LC/MS	–	0.63	<12.1	200	[7]
LLE	LC/UV	2–20	2.0	<5.3	100	[8]
SPE	LC/UV	0.05–35	0.05	≤6.0	500	[9]
LLE	LC/UV	0.25–15	0.25	≤5.0	200	[10]
SPE	LC/UV	0.16–20	0.16	≤5.0	500	[11]
In-tube SPME[c]	LC/UV	0.1–100	0.1	≤1.7	200	–

[a] SPE, solid-phase extraction.
[b] LLE, liquid–liquid extraction.
[c] Developed method.

corresponding correlation coefficient were $y = 0.1227x + 0.0264$ and $r^2 = 0.998$, respectively.

According to FDA guidelines [21], the LOQ (0.1 μg/mL) was determined as the lowest concentration of the calibration curve that can be measured with acceptable accuracy and precision (variation coefficient was lower than ≤20%).

Accuracy and inter-assay precision of the in-tube SPME/LC method were evaluated at three levels (high, medium, and low). As can be observed in Table 1, the accuracy of the method ranged from 80 to 93%, and the inter-assay precision assays presented coefficient of variation ≤1.7%. The data quality control samples for single batch are in agreement with the acceptance criteria.

The in-tube SPME/LC method was compared with conventional methods (liquid–liquid extraction and solid-phase extraction) employed for rifampicin analysis in plasma samples (Table 2). According to Table 2, the in-tube SPME/LC method presented many practical advantages over other methods described in the literature, including automation of the extraction process, small sample volume (200 μL), wide linear range, lower inter-assay coefficient variation, and multiple reuse of the capillary. In this work, the robustness of the polyethylene glycol capillary was evident from the fact that it could be reused for 200 times without significant loss of extraction efficiency.

5. Clinical application of the in-tube SPME/LC method

The effectiveness of the in-tube SPME/LC method for rifampicin determination was evaluated by analyzing plasma samples of six patient undergoing treatment for tuberculosis ($n = 3$). Fig. 4c illustrates the chromatogram of a plasma sample collected from a tuberculosis patient treated chronically with 600 mg rifampicin/day. The determined average concentration in patient plasma samples ranged from 1.7 to 7.0 μg/mL. These plasma levels are in agreement with literature data [2].

6. Conclusion

The in-tube SPME/LC method allowed automated continuous sample preparation (extraction, concentration, desorption, and injection of analytes) and minimized the analysis time as well as the volumes of organic solvent and biological fluid.

According to the analytical validation, the in-tube SPME/LC method is precise, accurate, and has adequate linear range for rifampicin determination in plasma sample for therapeutic drug monitoring.

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