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Research Article
Analysis of Rifampicin in Dried Blood Spots Using High Performance Liquid Chromatography

Yahdiana Harahap, Ghina Ashiila Amalia and Baitha Palanggatan Maggadani

Bioavailability and Bioequivalence Laboratory, Faculty of Pharmacy, Universitas Indonesia, UI Depok Campus, 16424 Depok, Indonesia

Abstract

Background and Objective: Rifampicin (RIF) is one of the first-line anti-tuberculosis drugs combined with isoniazid in fixed dose combination (FDC) form. Tuberculosis patient using RIF has been correlated with treatment failure because of low blood drug concentrations. Therefore, the application of therapeutic drug monitoring (TDM) is recommended. Besides the conventional venipuncture bio-sampling method, dried blood spots (DBS) offer several potential benefits, which increase the patient comfort. The main objective of this research is to obtain an analysis method of RIF in DBS. **Materials and Methods:** A simpler analytical method was developed and validated to quantify RIF in DBS samples using a high performance liquid chromatography (HPLC). A linear regression was used as the statistical analysis method. **Results:** The optimum chromatographic condition was obtained using C-8 (Waters, Sunfire™ 5 µm; 250×4.6 mm) with the mobile phase consisted of 50 mM ammonium acetate buffer pH 4.5-acetonitrile-methanol (40:30:30), flow rate was 0.5 mL min^{-1}, column temperature was 40°C and was detected at 261 nm. The time of analysis was 16 min and cilostazole was used as an internal standard. The DBS extraction was done by protein precipitation technique using acetonitrile:methanol (1:4 v/v%) with 1000 µL as the volume. The method was linear at concentration range of 1.0-30.0 µg mL^{-1} with r>0.9984. **Conclusion:** The analysis method was fulfilled the acceptance criteria of the European Medicines Agency (EMA) Bio-analytical Method Validation Guideline, 2011 and it is applicable for TDM of RIF.

Key words: Isoniazid, rifampicin (RIF), therapeutic drug monitoring (TDM), tuberculosis, cilostazole

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Corresponding Author: Yahdiana Harahap, Bioavailability and Bioequivalence Laboratory, Faculty of Pharmacy, Universitas Indonesia, UI Depok Campus, 16424 Depok, Indonesia Tel: +62818222192

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tuberculosis (TB) is the most common infectious disease caused by *Mycobacterium tuberculosis*[1]. TB remains as a major global health problem, especially in developing countries. In 2015 according to the World Health Organization (WHO), it was estimated up to 9.6 million TB cases had caused 1.2 million deaths in 2014. Indonesia has become one of the highest number of TB patients in the world with the 395 per 100,000 population in 2015[2].

Rifampicin (RIF) is the first line anti-TB drug, along with isoniazid, ethambutol and pyrazinamide. RIF is a semi-synthetic derivative antibiotics, which inhibits DNA-dependent RNA polymerase (RNAP) of mycobacteria and other microorganisms by forming a stable drug-enzyme complex[3]. The chemical structure of RIF is shown in Fig. 1.

RIF is combined with isoniazid in fixed dose combination (FDC) tablet form, that must be consumed for 4 months[4]. A study conducted by Fahimi *et al.*[5] indicated that 92.5% among 60 pulmonary TB patients had low concentrations of RIF. Low anti-TB drug concentrations not only leads to treatment failure, but may also cause drug resistance[5]. Thus, monitoring RIF in plasma helps to improve the effectiveness of therapy. The effective therapeutic range of RIF is between 8-24 μg mL^{-1} [6].

Prior to determining analytes in biological matrix, especially blood, bio sampling of the biological matrix should be conducted. A conventional bio-sampling venipuncture method can leads to patient inconvenience. Innovative dried blood spots (DBS) sampling technique for therapeutic drug monitoring (TDM), can be done by spotting wet blood onto absorbent paper or other paper materials and allowed to dry. The DBS method offers several potential benefits such as low blood volume, simplified blood sample collection, convenient sample storage and transfer and stabilize certain analytes or metabolites[7,8]. A study conducted by Allanson *et al.*[9], determined RIF in DBS using high performance liquid chromatography (HPLC) had found a correlation between the concentrations of RIF in plasma and blood spots ($r^2 = 0.92$). Another study performed by Vu *et al.*[10], determined RIF and clarithromycin in DBS using liquid chromatography tandem mass spectrometry (LC-MS/MS), provided 0.15 μg mL^{-1} as the lowest concentration in calibration curve.

However, the described method took a longer time for analysis and pre-treatment steps, also did not conduct the selectivity towards isoniazid, which was present along with RIF in FDC tablet form[9]. Another study provided higher sensitivity, but LC-MS/MS is still rarely found in Indonesia[10]. This research was conducted to develop the validated analytical method for providing a shorter time of analysis and pre-treatment steps, simpler chromatographic conditions and conducting selectivity towards isoniazid. This method can be useful for TDM of RIF in TB patients, which is also consume isoniazid in FDC tablet form.

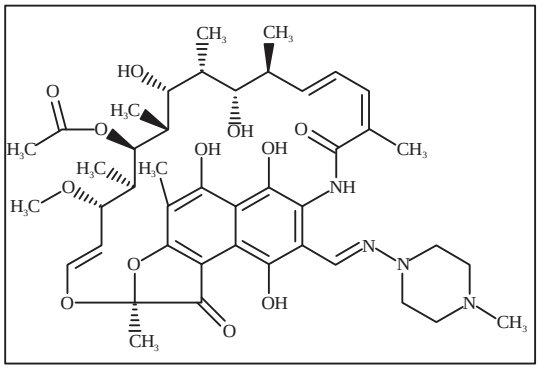

Fig. 1: Chemical structure of rifampicin

MATERIALS AND METHODS

Materials and reagents: Rifampicin (U.S. Pharmacopeia, Rockville, U.S.), isoniazid (Zhejiang Jiangbei Pharmaceutical Co. Ltd, Taizhou, China), cilostazole as an internal standard (Asia Chemical Industries Ltd, Beer-Sheva, Israel), HPLC grade acetonitrile, HPLC grade methanol, ammonium acetate and sodium dihydrogen phosphate Merck, aquabidest and ascorbic acid from Brataco and PerkinElmer 226 as the DBS card (PerkinElmer, Massachusetts, U.S.). The study was carried out from January until May, 2017.

Instruments and conditions: High performance liquid chromatography (Shimadzu, LC-20AD) equipped with pump, degasser, column oven, autosampler, UV-V is detector (Shimadzu, SPD-20A) and data processor (Lab Solutions). C-18 (Waters, Sunfire™ 5 μm; 250×4.6 mm), C-8 (Waters, Sunfire™ 5 μm; 250×4.6 mm), spectrophotometer UV (Jasco), evaporator (Turbo Vap LV), ultrasonicator (Elmasonic), pH meter (EUTECH), vortex (Maxi Mix II), centrifugator (Digisystem), microcentrifugator (Spectrafuge 16M) and micropipette Eppendorf (Socorex).

This study was conducted using C-8, 5 μm, 250×4.6 mm in HPLC method. The mobile phase consisted of ammonium acetate buffer 50 mM, pH 4.5-acetonitrile-methanol (40:30:30) with an isocratic elution. Injection volume was 20 μL. Detection was carried out at 261 nm, the column temperature was at 40°C and the flow rate was 0.5mL min^{-1}.

Preparation of the samples

Preparation of standard solutions and quality control solutions: A two separated stock solutions of RIF 1.0 mg mL^{-1} (1000 ppm) were prepared in methanol containing 0.5 mg mL^{-1} ascorbic acid. These stock solutions were used to prepare the calibration curve and quality control (QC) samples. A standard solutions of isoniazid and cilostazole 1.0 mg mL^{-1} (1000 ppm) was prepared in methanol and diluted to obtain solutions in certain concentrations. The standard solutions were stored at -20°C until being used.

Preparation of DBS samples: The packed whole blood with different hematocrit values obtained from Indonesian Red Cross. RIF spiked blood samples were obtained by mixing the standard RIF solutions with blank blood to yield final concentrations of 1.00, 6.00, 12.75, 18.00, 22.50 and 30.00 µg mL^{-1} for the calibration curve. As for the QC samples, were obtained by mixing the quality control solutions with blank blood to yield final concentrations at QC level, low (QCL), medium (QCM) and high (QCH). About 30 µL blood sample was spotted onto the DBS card using a micropipette. The spotted cards were allowed to dry for 3 h in room temperature, protected from light. After that, the DBS cards were stored separately in sealed plastic bags with desiccant sachets.

Development of RIF analytical condition in the presence of isoniazid using HPLC: Twenty microliters mixture of 50.0 µg mL^{-1} RIF, 50.0 µg mL^{-1} isoniazid and 50.0 µg mL^{-1} cilostazole was injected to the HPLC system with C-18 as the initial column used, then the chromatography responses were observed. The optimization was started by choosing the wavelength for the analysis. The optimization of mobile phase was conducted using the mixtures between ammonium acetate buffer, methanol and acetonitrile. Then optimization of the mobile phase composition was in the ratio of 60:20:20, 50:40:10, 50:20:30 and 40:30:30 followed with the column type using C-8. The pH of the buffer was optimized in 4.5, 5.0 and 5.5. The flow rate of the mobile phase was optimized in 0.5, 0.7 and 0.9 mL min^{-1}. The column temperature was optimized on 35, 40 and 45°C.

System suitability test: After obtaining the optimum conditions for RIF analysis in the presence of isoniazid, then the mixture of RIF solutions, isoniazid solutions and internal standard solutions was injected with 6 times repetition. The coefficient of variation (CV) requirements or the repetitive time retention the area and the tailing factor of each compounds that would be measured as PAR should be <2.0%.

Development of the DBS extraction: The extracting method between liquid- liquid extraction and protein precipitation were compared. After choosing the optimum extraction method, different extracting solvent including mixtures of methanol and acetonitrile with various compositions were observed at 500, 750, 1000 and 1500 µL. The time of sonication was also optimized for 5, 10 and 15 min.

Extraction procedure used from DBS for validation: Protein precipitation method was conducted by cutting the whole spotted area in DBS card and putting it into a 1.5 mL microtube. Extracting solution contained acetonitrile-methanol (1:4 v/v%, 1 mL) and 50 µL of internal standard solution with 10 µg mL^{-1} as the concentration were added. The sample was vortexed for 2 min and sonicated for 15 min at 30°C. Then, the sample was centrifuged at 10000 rpm for 5 min. The supernatant was evaporated until dry using nitrogen gas at 35°C and the residue was reconstituted in 200 µL mobile phase. The sample was vortexed for 10 sec and sonicated for 5 min to dissolve the analytes. The sample was transferred to a vial, then centrifuged 3000 rpm for 5 min to obtain clear solutions. Twenty microliters of sample was injected to the HPLC system with the chosen chromatography condition.

Method validation: In this research, the method was validated referring to European Medicines Agency (EMA) guideline for Bio-analytical Method Validation, 2011. A full validation of RIF analytical method in dried blood spots was conducted in term of selectivity, carry over, lower limit of quantification (LLOQ), linear calibration curve, accuracy, precision, dilution integrity and stability parameter[11]. In addition to these validation guideline, recovery test was performed. The validation was performed with maximum tolerated bias (% diff) and CV of 20% for the LLOQ and 15% for the other validated concentrations.

RESULTS AND DISCUSSION

Method development: The maximum wavelength obtained for the analysis was at 261 nm. The buffer type phosphate buffers showed poor peak shapes. Therefore, ammonium acetate buffer was chosen because it gives the best buffering ranges between 3.8<pH<5.8[12]. Compared with C-18, C-8 column has shorten the time of analysis (from 20-18 min) because it lowers the analytes affinity towards the stationary phase. The pH value of the buffer at 4.5 fasten the analytes elution compared to at 5.0 and 5.5 buffer pH value. So, the analysis time needed was 16 min with 5, 10 and 13 min time

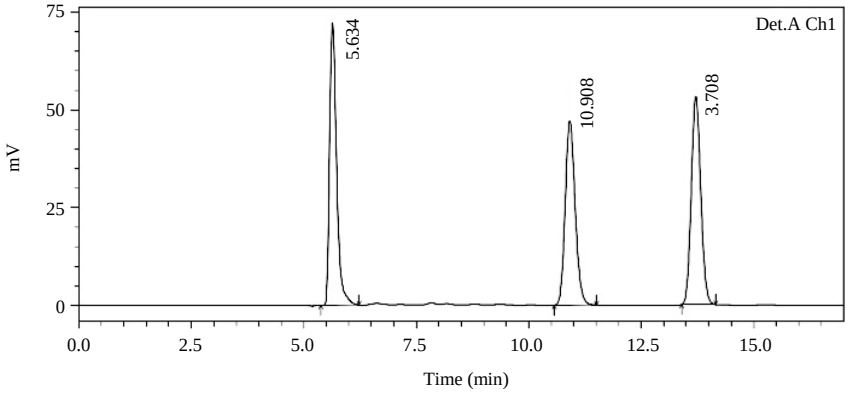

Fig. 2: Chromatogram of system suitability test

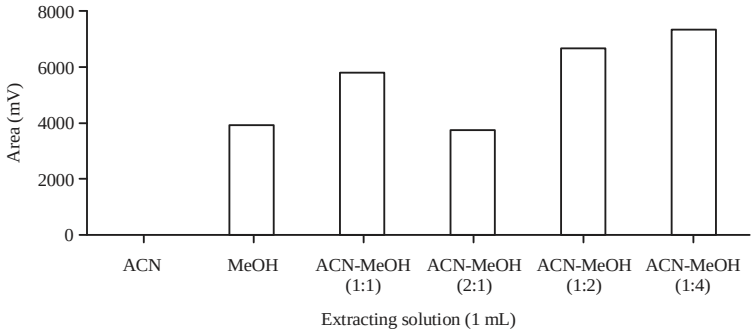

Fig. 3: Extracting solution optimization of RIF in acetonitrile (ACN) and methanol (MeOH)

retention of isoniazid, RIF and internal standard, respectively. The response area of RIF decreased as much as 28.5 and 43.5% for 0.7 and 0.9 mL min^{-1}, respectively. Further more the flow rate affected the column pressure which can increase the column pressure. The 0.5 mL min^{-1} was used as the flow rate in order to obtain the highest response with lower pressure. The column temperature that was used did not affect the analysis significantly, but the higher the column temperature can decreases the column pressure. Meanwhile, column temperature at 45°C RIF could be degraded[13].

The described analytical condition provides the highest area, the fastest relative retention time, tailing factor (T_f) that was almost 1, resolution (R)>2, the highest theoretical plate (N) and height of packing equivalent to a theoretical plate (HETP) close to 0. Also, isoniazid that present in the analysis did not interfere the results. The chromatogram result obtained is shown in Fig. 2.

The DBS at low level was used to evaluate the process efficiency from different extraction methods. The analytical method of RIF determination in DBS had been validated previously[9]. However, the method described using 3 mL of acetonitrile as the extracting solution. Besides being too excessive, it also took longer time (approximately 50 min) for the extracting solution to be evaporated at ambient temperature until dry.

The developed DBS of RIF analytical method mainly focused on the extraction procedure. The use of micropipette in spotting the spiked blood and cutting the whole spotted area can minimize the negative effects from the variation of hematocrit values, the spotted volume and the DBS paper type[8]. The liquid-liquid extraction method using ether and ethyl acetate were found not effective. This could occur because the extracting solvent was not strong enough to dissolve the analytes[14]. Also, RIF has a poor solubility in ether[15]. Meanwhile, mixtures of methanol and acetonitrile were able to dissolve the analytes. Therefore, the protein precipitation method was chosen as the extraction method. Mixtures of acetonitrile-methanol with 1:4 v/v%, 1000 µL was found as the optimum extracting solvent. It provided the highest area compared to the other compositions. The time of sonication needed to accelerate the extraction procedure was 15 min. The results of extracting solution optimization is graphically shown in Fig. 3.

Method validation

Determination of the LLOQ and calibration curve: The previous study had 1.5 µg mL^{-1} as the LLOQ concentration[9]. In this study, the LLOQ concentration of RIF was 1.0 µg mL^{-1} with the % CV value was 6.22% and % diff value was ranged between -0.92 to 15.68. The results still met the requirements. A calibration curve was made from seven-level concentrations of RIF in DBS. The concentrations of RIF in calibration curve ranging from 1.0-30.0 µg mL^{-1}, which was linear with the linear regression y = 0,0043+0,0135x and the correlation coefficient (r>0.9984). The % CV and % diff obtained results from the calibration curve met the requirements. The calibration curve of RIF is shown in Fig. 4.

Selectivity: Selectivity test was conducted in the DBS spotted with blank and LLOQ concentrations by using whole blood from six different sources. The response of interfering components in the time retention were 3.93-13.33% and 0.12-0.28% for the analyte and the internal standard, respectively. The values were less than ±20% of the LLOQ for the analyte and ±5% for the internal standard. Based on the selectivity test result, it is concluded that the analytical method of RIF in the presence of isoniazid fulfilled the selectivity parameter. Isoniazid that present in the matrix also showed no interference. The chromatogram of blank DBS and LLOQ obtained are shown in Fig. 5 and 6, respectively.

Carry over: Study showed no carry over effect in the DBS spotted with blank after injecting the highest concentration (ULOQ) of RIF. The carry over percentage fulfilled the requirement with the average percentage was 11.88%.

Accuracy, precision and recovery: Accuracy and precision test was conducted in within-run and between-run on 4 different

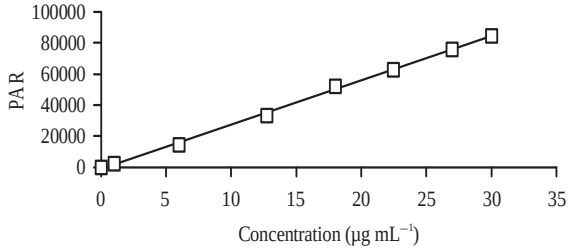

Fig. 4: Calibration curve of rifampicin. PAR: Photosynthetically active radiation

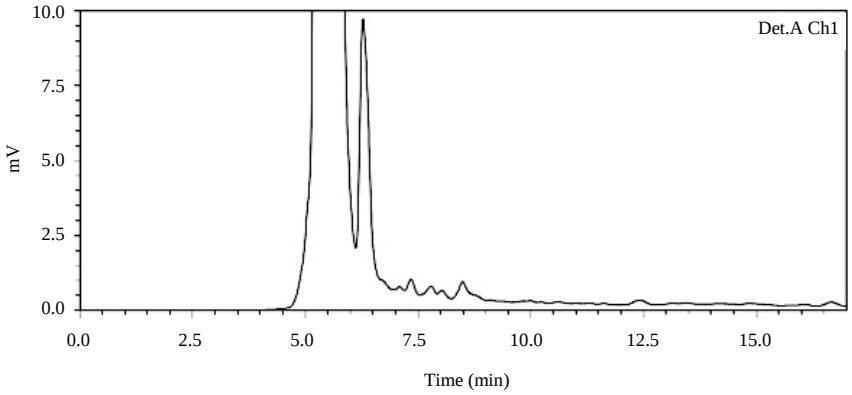

Fig. 5: Chromatogram of blank DBS

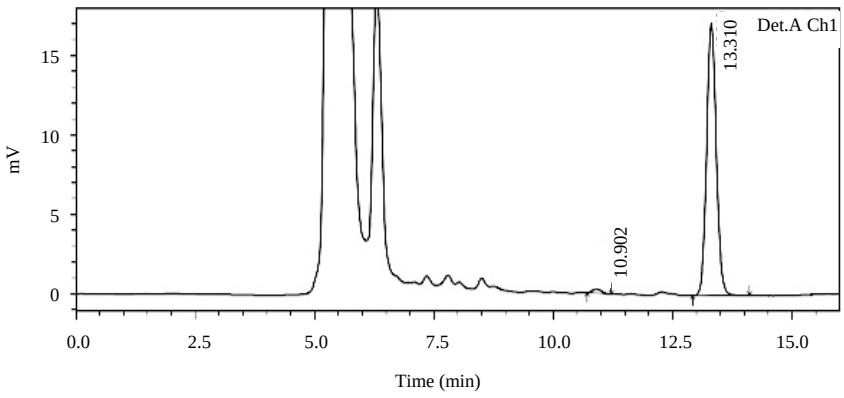

Fig. 6: Chromatogram of LLOQ

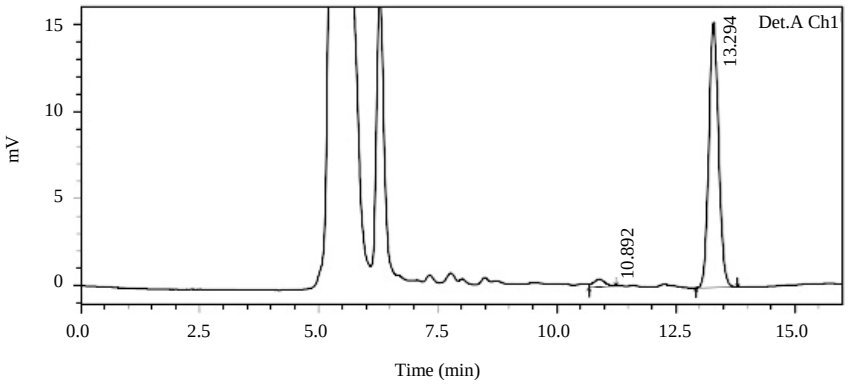

Fig. 7: Chromatogram of QCL

Table 1: Accuracy and precision within-run

	Day 1			Day 2			Day 3		
RIF concentrations (μg mL^{-1})	Measured concentrations (μg mL^{-1})	CV (%)	Diff. (%)	Measured concentrations (μg mL^{-1})	CV (%)	Diff. (%)	Measured concentrations (μg mL^{-1})	CV (%)	Diff. (%)
1.00	1.03	8.36	3.44	1.08	1.03	8.02	1.19	1.04	19.37
	0.97		-3.03	0.87		-13.01	1.14		13.90
	0.82		-17.88	1.16		15.61	0.90		-10.16
	0.98		-2.38	1.07		6.70	1.12		11.53
	0.98		-1.74	0.97		-2.84	0.86		-13.52
3.00	2.61	1.10	-13.00	3.06	5.14	1.96	3.36	8.15	12.06
	2.64		-12.04	2.70		-9.94	2.75		-8.50
	2.58		-13.97	2.75		-8.38	2.95		-1.77
	2.57		-14.36	2.76		-7.90	3.27		9.11
	2.62		-12.61	2.75		-8.32	3.19		6.31
12.75	13.75	5.88	7.82	12.35	5.45	-3.13	10.84	6.76	-14.97
	12.97		1.74	13.91		9.07	10.89		-14.58
	12.01		-5.78	13.06		2.42	11.09		-12.98
	11.96		-6.20	13.75		7.86	11.31		-11.30
	12.92		1.35	14.15		10.98	12.70		-0.37
22.50	20.08	9.88	-10.75	24.60	9.23	9.35	22.96	4.20	2.03
	21.30		-5.33	20.73		-7.85	22.27		-1.04
	21.25		-5.55	24.13		7.22	21.46		-4.63
	25.14		11.75	24.41		8.49	20.77		-7.69
	24.47		8.74	20.37		-9.48	22.80		1.34

RIF: Rifampicin

concentrations, that are LLOQ, QCL, QCM and QCH. The accuracy and precision within-run results are shown in Table 1. The % CV in the between run test were11.40, 9.29, 9.03 and 7.76% for LLOQ, QCL, QCM and QCH, respectively. It can be concluded that the method was accurate and precise. The recovery was conducted to compare the response area in DBS with the standard RIF in solutions. The average recovery obtained for RIF in the DBS was 66.89%. The chromatogram of QCL, QCM and QCH obtained are shown in Fig. 7-9, respectively.

Dilution integrity: Dilution integrity was conducted to determine the accuracy, precision and reliability of the dilution process in bioanalytical process. Dilution integrity was analyzed using higher concentration of ULOQ (2x QCH), then diluted into ½ and ¼ of its concentration. The obtained % CV results were 5.55, 10.31 and 7.11% for 2xQCH, ½QCH and ¼ QCH, respectively. Then, the obtained % diff results were ranged between -14.58% to +14.64%, so it can be concluded that dilution process during analysis was accurate, precise and reliable.

Stability: Stability was analyzed by using QCL and QCH samples, each with 3 replications. The short-term stability in DBS, samples were stored in room temperature and the stability was observed in 0, 6 and 24 h. The obtained results

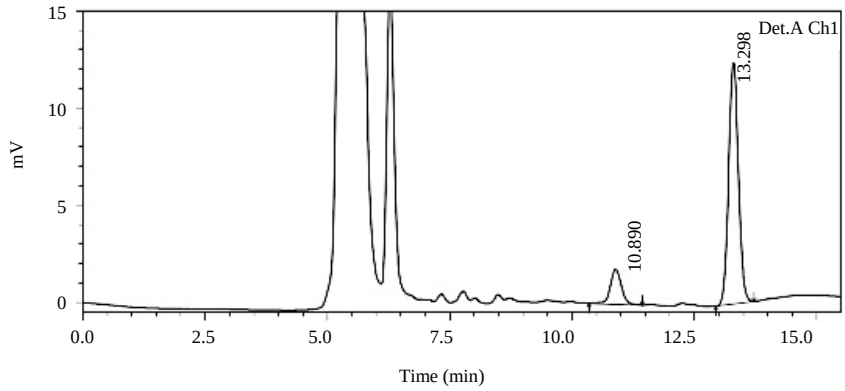

Fig. 8: Chromatogram of QCM

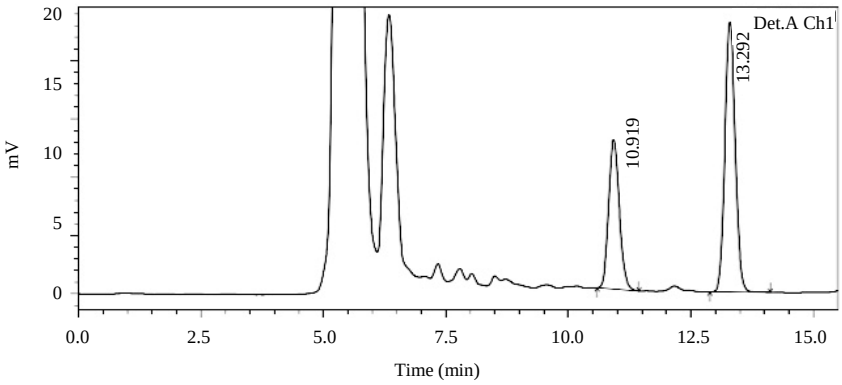

Fig. 9: Chromatogram of QCH

Table 2: RIF long-term stability in the DBS (dried blood spots) in room temperature

QCL			QCH		
3 (μg mL^{-1})	CV (%)	Diff (%)	22.5 (μg mL^{-1})	CV (%)	Diff (%)
0 days	0.69	8.80	0 days	2.67	3.80
		7.82			8.61
		9.29			3.67
7 days	4.23	-7.48	7 days	3.32	1.93
		-6.45			3.84
		-0.04			8.68
14 days	3.12	2.15	14 days	3.88	1.68
		2.14			1.96
		7.76			-4.87
40 days	12.84	12.54	40 days	1.63	11.29
		6.63			14.76
		-12.56			11.96

QCL: Quality control at low level, QCH: Quality control at high level

showed that RIF in DBS were stable in room temperature atleast for 24 h. For the long-term stability, samples were stored in room temperature for 40 days and analyzed at day-7, day-14 and day-40. The obtained results for the long-term stability are shown in Table 2. It is concluded that RIF in DBS were stable to be stored in room temperature at least for 40 days.

The obtained results from the autosampler stability test ranged between -14.36 to 8.80%. Those results showed that the prepared RIF from DBS were stable in minimum 24 h in autosampler.

The long-term stability of the RIF and cilostazole standard solutions, which were stored in -20°C and analyzed at day-7, day-14 and day-28. For long-term stability, the obtained

results of % diff for RIF solutions ranged between -14.32 to 0.59% and % diff for cilostazole solutions ranged between -7.28 to 0.00% for 28 days in -20°C. Therefore, it can conclude that RIF and cilostazole stock solutions, which were stored in -20°C, can be used atleast for 28 days.

Compared with the method reported in previous research for RIF in DBS using HPLC, this method has higher sensitivity, which could be used for TDM in TB patients with a more convenient method. Provided samples are taken at a time when plasma concentrations of RIF are at maximum (approximately 2 h after dosing). A good correlation between plasma and DBS methods had been shown to be linear[9].

CONCLUSION

The developed analytical method was valid and linear ranged from 1.00-30.00 μg mL^{-1} for RIF with higher sensitivity, simpler chromatographic condition and preparation method and a few extracting solution with higher recoveries. All the parameters fulfilled the acceptance criteria of the EMA Bio-analytical Method Validation Guideline, 2011. The samples can be stored up to 40 days in room temperature, therefore, this method is suitable for conducting TDM of RIF in remote areas.

SIGNIFICANCE STATEMENT

This study discovers a validated method with a simpler chromatographic condition and preparation method to analyze rifampicin in dried blood spots with the presence of isoniazid. This can be beneficial for therapeutic drug monitoring of rifampicin in tuberculosis patients, which also consume isoniazid in FDC tablet form. This study will help the researcher to conduct a simpler method to determine rifampicin in tuberculosis patients in purpose of therapeutic drug monitoring with a more convenient method.

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