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Determination of rifampicin and its main metabolite in plasma and urine in presence of pyrazinamide and isoniazid by HPLC method

R. Panchagnula *, A. Sood, N. Sharda, K. Kaur, C.L. Kaul

Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research, Sector 67, S.A.S., Nagar, Punjab, 160 062, India

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Abstract

A reversed phase HPLC method is described for the simultaneous estimation of rifampicin and its major metabolite desacetyl rifampicin, in the presence of isoniazid and pyrazinamide, in human plasma and urine. The assay involves simple liquid extraction of drug, metabolite and internal standard (rifapentine) from biological specimens and their subsequent separation on a C_{18} reversed phase column and single wavelength UV detection. In plasma as well as in urine samples, all the three compounds of interest eluted within 17 min. Using methanol–sodium phosphate buffer (pH 5.2; 0.01 M) (65:35, v/v) as mobile phase under isocratic conditions, it was established that isoniazid, pyrazinamide and ascorbic acid (added to prevent oxidative degradation of analytes) did not interfere with the analyte peaks. Recoveries (extraction efficiency) for drug were greater than 90% in both plasma and urine, whereas for metabolite the values were found to be 79 and 86% in plasma and urine, respectively. The plasma and urine methods were precise (total coefficient of variation ranged from 5 to 23%) and accurate (-7 to 5% of the nominal values) for both the analytes. Individual variance components, their estimates and their contribution to the total variance were also determined. Using the same method, unknown samples supplied by WHO were assayed and good correlations were obtained between the found and intended values. The method developed proved to be suitable for simultaneous estimation of rifampicin and desacetyl rifampicin in plasma and urine samples.

Keywords: Reversed phase chromatography; Rifampicin; Desacetyl rifampicin; Rifapentine; Simultaneous determination

1. Introduction

Tuberculosis is one of the major communicable diseases in the developing countries. The therapeutic potential of rifampicin (RIF) in tuberculosis is well recognized due to its unique ability to

* Corresponding author. Tel: +91 172 677226, +91 172 673848; fax: +91 172 677185; e-mail: niper@chd.nic.in

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kill semi dormant tubercule bacilli (*Mycobacterium tuberculosis*), when they undergo sporadic bursts of metabolism and growth [1–3]. It is categorized amongst first line agents including isoniazid (INH), pyrazinamide (PYR), ethambutol (ETB) and streptomycin (STP) which are used in combination as effective therapy for all forms of disease caused by *M. tuberculosis*. WHO recommends a 6-month regimen comprising RIF, INH, PYR and ETB which are given together for the first 2 months followed by RIF and INH therapy for the next 4 months. RIF is mainly eliminated in the bile and then reabsorbed, hence, enterohepatic circulation ensues. During this time the drug is progressively deacylated into its microbiologically active metabolite, 25-desacetyl rifampicin (DRIF) which is less absorbable as compared to the parent drug [4].

Earlier, RIF in plasma/serum was quantified by microbiological methods [5–7] but these methods lacked precision and selectivity [8,9]. In the recent past, several HPLC procedures have been reported in the literature for quantitative estimation of RIF and its metabolite, in serum/plasma or urine [10–17]. But many of these methods suffer from limitations such as lengthy and tedious procedures, high plasma/serum sample volumes required, large quantities of solvents involved etc. The present study was undertaken with the objective to develop and validate a simple, sensitive HPLC assay procedure for simultaneous determination of RIF, DRIF in biological fluids in the presence of INH and PYR so as to overcome the limitations of previously reported methods.

2. Experimental

2.1. Materials

RIF, DRIF and rifapentine (RPT) were gift samples from WHO Global Tuberculosis Program (supplied by Dr Gordon Ellard, London University). PYR and INH were gift samples from Lupin laboratories, India. All other reagents were either of HPLC or AR grade procured from Loba Chemie, E. Merck (India) or Ranbaxy. Triple distilled water filtered through 0.45 μm membrane filter was used in all the experiments.

2.2. Instrumentation

Waters HPLC system consisting of two 510 pumps, one 600 pump, 717 autosampler, 996 PDA detector (for method development studies) and 486 tunable UV detector was used in the study. Millennium software (version 2.1) was used for data acquisition and processing. Other instruments used included Mettler electronic balance AG 245, Branson 3210 sonicator, Heraeus Centrivac and Biofuge-13, Nichipet from Nichiryo and microlitre syringes from Hamilton.

2.3. Column

Analytical column used was reversed phase Nova-Pak C_{18} (250 × 4 mm i.d., 4 μm), supplied by Waters Associates.

2.4. Mobile phase

Various combinations of organic solvents (methanol, acetonitrile) and aqueous modifier [sodium phosphate buffer, 0.01 M; pH adjusted to 5.2 with 2% *o*-phosphoric acid] were tried as mobile phase. The final mobile phase composition optimized was methanol–sodium phosphate buffer (pH 5.2; 0.01 M) (65:35, v/v), which, at a flow rate of 1 ml min^{-1}, resulted into well resolved peaks for parent drug, its metabolite and internal standard in the presence of INH and PYR. The mobile phase was sonicated for 10 min after filtering through Millipore filter (pore size 0.45 μm) and used. Peaks for RIF, DRIF and RPT resolved within a maximum run time of 17 min (Fig. 1).

2.5. Sample preparation

Calibration stock solutions of RIF, DRIF and RPT were prepared in methanol whereas, PYR and INH stock solutions were prepared in water. Calibration stocks of RIF and DRIF (1 mg ml^{-1}) were suitably diluted to give working stock solutions of 100 μg ml^{-1} each and from these working stock solutions, calibration standards were prepared in plasma (2–20 μg ml^{-1} of RIF and 1–5 μg ml^{-1} of DRIF) and urine (20–200 μg

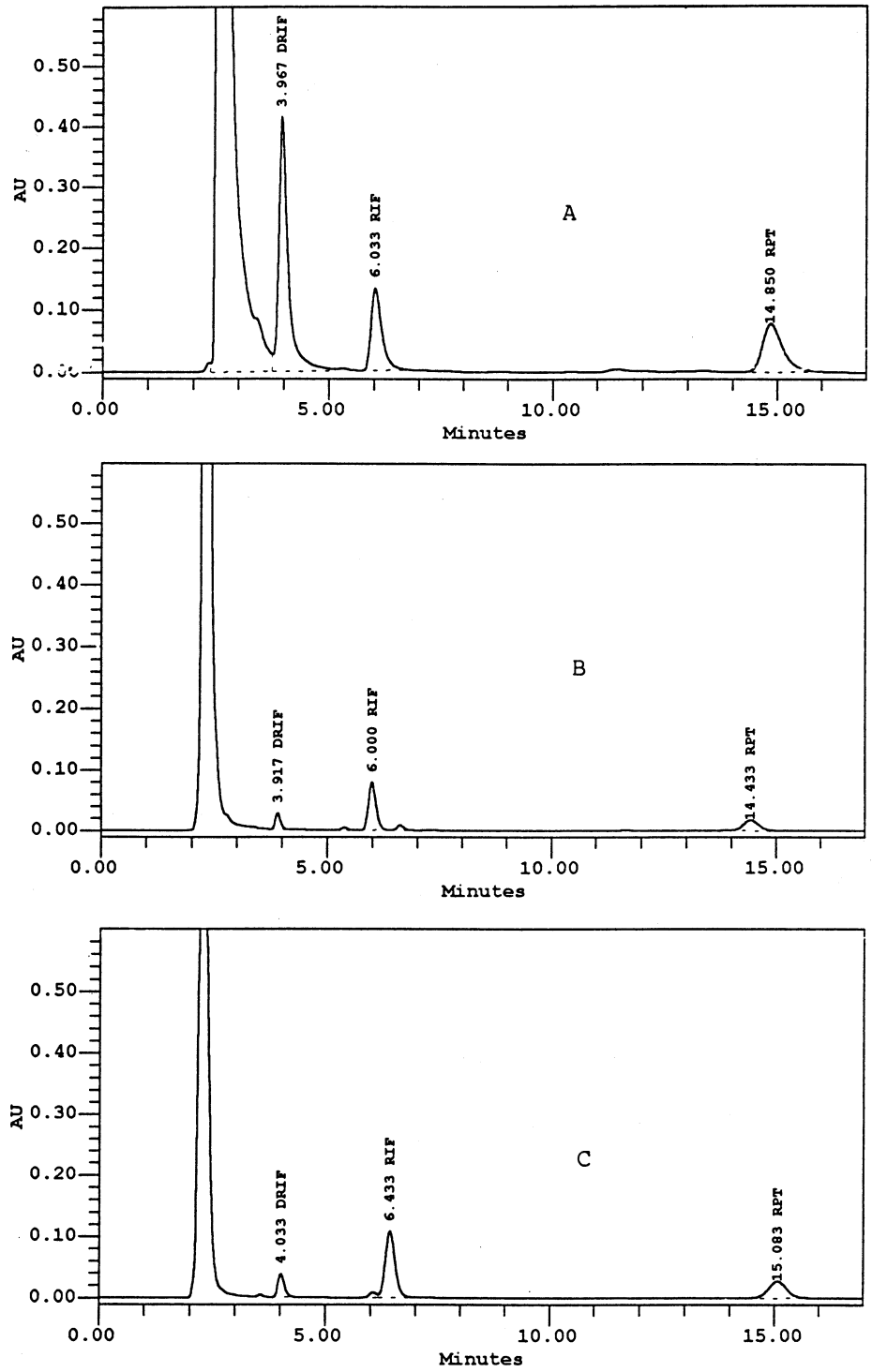

Fig. 1. Representative chromatograms of (a) Rifampicin (RIF), desacetyl rifampicin (DRIF)and rifapentine (RPT)in the presence of pyrazinamide, isoniazid and ascorbic acid (0.5 mg ml^{-1} each); (b) Blank human plasma spiked with rifampicin (17.6 μg ml^{-1}), desacetyl rifampicin (5.2 μg ml^{-1}) and rifapentine (1.0 μg ml^{-1}); (c) Blank human urine spiked with rifampicin (161.08 μg ml^{-1}), desacetyl rifampicin (47.27 μg ml^{-1}) and rifapentine (10.0 μg ml^{-1}). The peaks are annotated with their respective names and retention times.

ml^{-1} of RIF and 10–50 μg ml^{-1} of DRIF). Calibration concentration ranges for the drug and metabolite were selected on the basis of their expected steady state concentration levels in plasma and urine. RPT was added as an internal standard to give a concentration of 10 μg ml^{-1} in plasma and 100 μg ml^{-1} in urine. Quality control (QC) samples were prepared by adding different amounts of RIF and DRIF to plasma/urine to give desired known concentrations within the calibration range. It was established that when PYR and INH were added along with RIF and DRIF to plasma/urine, the drugs (PYR and INH) co-eluted with plasma/urine artifacts and didn't interfere with RIF, DRIF or internal standard peaks (Fig. 1). Hence, in final plasma/urine samples these drugs were not added. In both urine and plasma samples, ascorbic acid, at a concentration of 0.5 mg ml^{-1}, was added to prevent oxidation of RIF and its metabolites during sample processing.

2.6. Extraction procedure

2.6.1. For plasma

For the optimization of volume of plasma needed for extraction, different volumes of plasma (50–300 μl) were tried and 100 μl volume of spiked plasma was found to be sufficient to give detectable signals for both the analytes at lowest concentration levels used in calibration standards. The extraction procedure adopted was as follows:

Different amounts of RIF, DRIF and internal standard (RPT) were taken into 1.5 ml eppendorf tubes from working stock solutions (amounts being calculated to give calibration concentrations in 100 μl of plasma). These mixtures were dried and mixed with 5 μl of methanol, then 95 μl of plasma was added and vortexed for 60 s. Spiked plasma was then extracted with 500 μl of methanol by vortexing for 3 min. The samples were centrifuged at 10000 rpm for 15 min, and 300 μl of supernatant was taken into another micro centrifuge tube and vacuum dried in centrivac. The residue thus obtained was reconstituted in 100 μl of mobile phase and 50 μl volume was injected. All the three analytes; drug, metabolite and internal standard, were detected at 254 nm.

2.6.2. For urine

Similar procedure was adopted for urine extraction. The only differences were (i) the concentration of drugs used was ten times as that in plasma for calibration standards (ii) in the final step of the extraction procedure, the dried residue was reconstituted in 500 μl of mobile phase and 50 μl volume of this was injected.

2.7. WHO test samples

Vacuum dried test samples containing varying unknown amounts of RIF and DRIF were received from Dr G. Ellard, London on behalf of Dr Elizabeth Taylor, WHO Global Tuberculosis Program. All the samples ($n = 30$ for reconstitution in plasma and $n = 15$ for reconstitution in urine) were received in triplicate and labeled appropriately so as to distinguish the samples intended to be reconstituted in plasma from those to be reconstituted in urine. The samples contained appropriate quantities of RIF and DRIF to give (upon reconstitution in 2 ml plasma/urine) final concentrations within the ranges of 1–20 and 1–5 μg ml^{-1} in plasma, and 10–200 and 10–50 μg ml^{-1} in urine for RIF and DRIF, respectively. The test samples also contained sufficient amounts of ascorbic acid, to give a final concentration of 0.5 mg ml^{-1} after reconstitution, to prevent the oxidation of RIF and DRIF.

2.7.1. Sample reconstitution and analysis

Test samples were reconstituted according to the procedure suggested by WHO. Each vacuum dried test sample was dissolved in 50 μl volume of methanol by agitating on a vortex mixer. Then 0.95 ml blank plasma/urine was added and after thorough agitation it was decanted into a fresh tube. To the original test sample tube, another 1 ml aliquot of plasma/urine was added and any solid residue remaining was dissolved by vortexing. These two plasma/urine extracts were then combined to provide the test sample for assay.

From the reconstituted samples, 100 μl aliquots were taken and then processed and analyzed as described for calibration standards in plasma and urine respectively.

3. Results and discussion

3.1. Selectivity and specificity

Fig. 1 shows that parent drug (RIF) is clearly separated from its metabolite (DRIF) in the presence of INH and PYR. The initial trials with various compositions of mobile phase (methanol and buffer) resulted in poor resolution of RIF and DRIF peaks. Finally, mobile phase composition was optimized to be methanol–sodium phosphate buffer (pH 5.2; 0.01 M) (65:35, v/v) without losing the selectivity and specificity of method. Plasma artifacts didn't interfere with the peaks of rifampicin and its metabolite. Similar observations were also seen in case of urine samples. In the initial experiments, it was established that PYR and INH peaks co-eluted with plasma/urine artifacts and didn't interfere with RIF, DRIF or internal standard peaks (Fig. 1). Hence, in final plasma/urine calibration standards and quality control samples these drugs were not added.

3.2. Extraction efficiency

The recoveries of RIF, DRIF and RPT from plasma and urine were determined by comparing the peak areas of extracted standards and those of the unextracted standards at different concentration levels over the relevant concentration range. Using this method, the mean recovery from plasma was found to be 91.73 and 79.09% for RIF and DRIF, respectively. Whereas, from urine, the values were found to be 96.24 and 86.22% for RIF and DRIF, respectively. Recoveries of RPT from plasma as well as from urine was found to be above 95%.

3.3. Calibration range and linearity

Calibration graphs were constructed simultaneously for both RIF and DRIF. The calibration curves (ratio of peak areas of drug or its metabolite to that of internal standard versus concentration) were linear in plasma in concentration range of 2–20 and 1–5 μg ml^{-1} for RIF and DRIF, respectively. Whereas for urine samples linear correlations were found in the concentration range of 20–200 and 10–50 μg ml^{-1} for RIF and DRIF, respectively. The correlation coefficient was always greater than 0.999. Table 1 lists the calibration curve parameters such as, slope, intercept and correlation coefficients.

3.4. Variance and variance components estimations

The data obtained was subjected to statistical treatment and the method described by Lee and McAllister [18] was adopted for determining percent relative standard deviation (%R.S.D.) values for calibration standards of RIF and DRIF in plasma and urine at three different concentration levels. Individual variance components estimations (intra-day, inter-day and inter-analyst) and their relative contribution to total variance were also calculated. Results are presented in Table 2. It was found that contribution of intra-day vari-

Table 1
Calibration curve parameters of Rifampicin (RIF) and desacetyl rifampicin (DRIF) in plasma and urine samples ($n = 4$–6; mean values are listed)

Drug	Slope	Intercept	r	Equation of line
Plasma				
RIF	0.127209	0.01217	0.9999	$y = 0.127209x + 0.01217$
DRIF	0.099855	0.00744	0.9992	$y = 0.099855x + 0.00744$
Urine				
RIF	0.013519	−0.027399	0.9995	$y = 0.013519x - 0.0274$
DRIF	0.010806	0.0005	0.9993	$y = 0.010806x + 0.0005$

r = correlation coefficient.

Table 2
Variance components estimates for calibration standards of Rifampicin (RIF) and desacetyl rifampicin (DRIF) in plasma and in urine at three different concentration levels

Drug and conc. level	Source of variance	Variance estimates	% of total variance	%R.S.D.
Plasma				
RIF, 2 μg ml^{-1}	Inter-analyst	7.88×10^{-5}	11.17	3.49
	Intra-day	4.5×10^{-4}	63.12	8.29
	Inter-day	1.8×10^{-4}	25.71	5.29
	Total	7.1×10^{-4}	100	10.43
RIF, 8 μg ml^{-1}	Inter-analyst	7.89×10^{-4}	7.19	1.71
	Intra-day	7.98×10^{-3}	72.82	5.44
	Inter-day	2.19×10^{-3}	19.99	2.85
	Total	1.09×10^{-2}	100	6.38
RIF, 20 μg ml^{-1}	Inter-analyst	1.73×10^{-1}	50.02	4.94
	Intra-day	1.45×10^{-2}	42.12	4.53
	Inter-day	2.72×10^{-3}	7.86	1.96
	Total	3.45×10^{-2}	100	6.98
DRIF, 1 μg ml^{-1}	Inter-analyst	9.27×10^{-6}	9.32	7.07
	Intra-day	5.52×10^{-5}	55.55	17.25
	Inter-day	3.49×10^{-5}	35.13	13.72
	Total	9.94×10^{-5}	100	23.15
DRIF, 2 μg ml^{-1}	Inter-analyst	3.61×10^{-4}	60.78	5.90
	Intra-day	1.42×10^{-4}	23.95	3.70
	Inter-day	9.06×10^{-5}	15.27	2.96
	Total	5.94×10^{-4}	100	7.57
DRIF, 5 μg ml^{-1}	Inter-analyst	4.35×10^{-4}	20.29	3.74
	Intra-day	1.59×10^{-3}	74.35	7.16
	Inter-day	1.15×10^{-4}	5.36	1.92
	Total	2.14×10^{-3}	100	8.31
Urine				
RIF, 20 μg ml^{-1}	Inter-analyst	3.22×10^{-5}	12.63	2.44
	Intra-day	1.56×10^{-4}	61.20	5.36
	Inter-day	6.68×10^{-5}	26.18	3.51
	Total	2.55×10^{-4}	100	6.85
RIF, 80 μg ml^{-1}	Inter-analyst	2.0×10^{-4}	1.68	0.09
	Intra-day	1.13×10^{-2}	94.73	6.75
	Inter-day	4.29×10^{-4}	3.59	1.31
	Total	1.19×10^{-2}	100	6.93
RIF, 200 μg ml^{-1}	Inter-analyst	3.19×10^{-4}	1.51	0.67
	Intra-day	2.06×10^{-2}	97.70	5.41
	Inter-day	1.67×10^{-4}	0.79	0.49
	Total	2.11×10^{-2}	100	5.47
DRIF, 10 μg ml^{-1}	Inter-analyst	2.81×10^{-5}	50.20	14.54
	Intra-day	2.61×10^{-5}	46.68	14.02
	Inter-day	1.74×10^{-5}	3.12	3.62
	Total	5.59×10^{-5}	100	20.52
DRIF, 30 μg ml^{-1}	Inter-analyst	3.89×10^{-4}	35.55	6.60
	Intra-day	2.8×10^{-4}	25.08	5.55
	Inter-day	4.31×10^{-4}	39.37	6.95
	Total	1.09×10^{-3}	100	11.08
DRIF, 50 μg ml^{-1}	Inter-analyst	6.13×10^{-5}	1.50	1.65
	Intra-day	3.06×10^{-3}	75.03	11.66
	Inter-day	9.57×10^{-4}	23.46	9.52
	Total	4.07×10^{-3}	100	13.46

Table 3
Percentage recovery of labeled Rifampicin (RIF) and Desacetyl rifampicin (DRIF) from plasma and urine quality control samples

RIF			DRIF		
Labelled (μg ml^{-1})	Calculated (μg ml^{-1})	%	Labelled (μg ml^{-1})	Calculated (μg ml^{-1})	%
Plasma					
6	5.56	92.67	1.5	1.53	102.0
10	9.85	98.50	2.5	2.42	96.80
14	14.77	105.5	3.5	3.62	103.43
18	18.72	103.98	4.5	4.62	102.67
Urine					
60	60.56	100.93	15	15.18	101.23
100	97.06	97.06	25	23.49	93.96
140	140.1	100.07	35	36.24	103.54

ance component to the total variance was relatively, always, higher in plasma as well as in urine. This was attributed to slight changes in the concentration of RIF and DRIF calibration stock solutions due to volatile nature of the solvent (methanol) used to prepare the stock solutions.

3.5. Accuracy

Accuracy of the analytical method was tested by analyzing QC samples of different known concentrations (within the calibration range) (Table 3). In case of plasma samples, recoveries were in the range of 93–106 and 97–103% of nominal values for RIF and DRIF, respectively. Whereas for urine samples, the recoveries were in the range of 97–101 and 94–104% of nominal values for RIF and DRIF, respectively.

3.6. WHO test samples results

After checking the recoveries of QC samples the same method was applied to the unknown samples supplied by WHO. As directed by WHO, 30 unknown test samples were assayed in plasma and other 15 test samples were assayed in urine (each test sample in triplicate). The individual concentrations of RIF and DRIF quantitated in all the samples were communicated to Dr G. Ellard. The results obtained were compared and correlated to the actual intended concentration values by determining the ratios of found to intended concentrations. In plasma as well as in urine, the ratios were found to be close to 1.0, for both RIF and DRIF, with R.S.D. remaining always below 10% (Table 4).

4. Conclusions

There are a number of reversed phase chromatographic methods reported in literature for the simultaneous estimation of RIF and DRIF in serum/plasma and urine. However, the methods suffer from certain limitations such as high volumes of plasma/serum or urine samples required, lengthy and cumbersome extraction pro-

Table 4
Correlation between found and intended concentrations of rifampicin (RIF), desacetyl rifampicin (DRIF) in unknown WHO samples-ratio of found/intended concentrations; mean ± S.D. (% R.S.D.)

Ratio of found/intended concentrations	
RIF	DRIF
Plasma ($n = 30$; in triplicate)	
0.845 ± 0.073 (8.6%)	0.931 ± 0.086 (9.3%)
Urine ($n = 15$; in triplicate)	
1.030 ± 0.089 (8.7%)	0.984 ± 0.073 (7.4%)

cedures, large quantities of solvents required, or sometimes, very complex mobile phase compositions. The discussed reversed phase HPLC method is simple, rapid, sensitive and reproducible for analysis of RIF and DRIF in plasma and urine. The method is suitable for selective estimations of the drug and metabolite, in plasma/urine matrix, in the presence of INH and PYR which are a part of combination therapy in tuberculosis. Various features of the developed method include low volumes of plasma or urine samples required for analysis (100 μl), simple and fast extraction procedure, simple mobile phase composition and single wavelength detection of analytes as well as internal standard (254 nm). This makes the method very rapid and economical, especially when a large number of samples are handled. At the same time, method holds the potential for its adoption for the analysis of rifapentine in plasma and urine samples without employing expensive column switching techniques [19].

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