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Short communication

Simultaneous determination of rifampicin, isoniazid and pyrazinamid by high performance thin layer chromatography

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1. Introduction

Fixed dose combinations containing rifampicin (RIF), isoniazid (INH) and pyrazinamid (PZA), are widely available on the market for the medication and management of tuberculosis, a chronic disease that has plagued mankind since the dawn of time [1]. The chemical names of RIF, INH and PZA are 3-[{(4-methyl-1-piperazinyl)-imino}-methyl] rifamycin; [5,6,9,17,19,21-hexahydroxy-23-methyoxy-2,4,12,16,18,20,22-1-heptamethyl-8-[*N*-(-methyl-1-piperazinyl)formimidoyl]-2,7-(epoxy entadeca[1,11,13]-trienimino)-naphtha[2,1-*b*]furan-1,11(2*H*)-dione21]acetate,4-pyridinecarboxylic acid hydrazide; and pyrazine-2-carboxamide respectively. Many methods for the determination of INH [2–8], PZA [9–11] and RIF [12] from capsules and tablets have been reported. However, simultaneous determinations of these drugs from fixed dose combinations in unit samples are rare. A reversed phase HPLC method has been reported by Gaitonde and Pathak [13] for the simultaneous determination of RIF, INH and PZA in unit doses. However, there is a serious disadvantage to this method because the use of tetrabutylammonium phosphoric acid buffer pH 3.0 shortens the column life.

High performance thin layer chromatography (HPTLC) is a more effective technique for simultaneous determination in single samples in routine analysis. The aim of the present investigation is to develop an HPTLC method for the simultaneous determination of RIF, INH and PZA using ethyl acetate:ammonia:ethyl alcohol:cyclohexane (20:9:4.5:5, v/v/v/v) as mobile phase on glass baked silica gel 60 F 254 plates. Quantitative estimation was accomplished by densitometric scanning with a UV–Vis detector.

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2. Experimental

2.1. Instrumentation

The following were used: a Camag Linomat IV sample applicator with Scanner II, 3.15 V Cats software and a twin trough chamber, Merck 60 F 254 HPTLC glass plates after washing with 0.01 M potassium dihydrogen phosphate buffer pH 5.0 and drying at 100°C and a Millipore filtration kit.

2.2. Materials

Standard INH and PZA were procured from IPCA Laboratories (Bombay, India). Standard RIF and its related substances, 25-desacetyl rifampicin, rifampicin quinone SV and 3-formyl rifampicin SV were obtained from Lupin Laboratories (Tarapur, Thane, India). Tablets containing RIF, INH and PZA were purchased on the commercial market.

2.3. Reagents and chemicals

Analytical-grade liquid ammonia, ethyl acetate, ethyl alcohol, cyclohexane, chloroform, methanol and potassium dihydrogen phosphate were supplied by S.D. Fine Chemicals (Thane, India).

2.4. Mobile phase

A mixture of ethyl acetate, ammonia (6.75 M), ethyl alcohol and cyclohexane (20:9:4.5:5, v/v/v/v) was taken in a separating funnel, shaken for 3 min and the two layers allowed to separate. The lower layer was decanted off and the upper layer was used as a mobile phase after drying over anhydrous sodium sulphate.

2.5. Standard stock solution

Accurately weighed 50 mg portions of each of the standard RIF, INH and PZA were transferred to a standard 50 ml volumetric flask, dissolved and diluted to the mark with a 60:40 mixture of chloroform and methanol. 2 ml of this solution was further diluted to 50 ml with a chloroform and methanol mixture. This solution was used as a standard stock solution.

2.6. Linearity and detector response

Varying amounts of standard stock solution containing 120–400 ng for RIF (3–10 μl) and 40–200 ng for PZA (1–5 μl) were applied to HPTLC glass plates in a 8 mm band with the help of a sample applicator.

The plates was saturated for 20 min and then developed up to 90 mm in a twin trough chamber containing ethyl acetate:ammonia (6.75 M):ethyl alcohol:cyclohexane (20:9:4.5:5, v/v/v) as a mobile phase.

Densitometric evaluation was done by a TLC scanner controlled by Cats 3.15 version software and absorbance was measured at two different wavelengths: 440 mm for RIF and 275 nm for INH and PZA using a deuterium (D2) lamp. Peak areas were recorded for all the tracks.

Calibration curves were constructed for RIF, INH and PZA by plotting areas (y axis) against the amount of drug in nanograms (x axis) (Fig. 1).

2.7. Determination of RIF, INH and PZA in pharmaceutical preparations

20 tablets were accurately weighed and powdered, an amount of the powder equivalent to the average weight of a tablet was taken in a 100 ml volumetric flask, 50 ml of a 60:40 mixture of chloroform:methanol was added and the flask was placed in an ultrasonic bath for 20 min and finally diluted to the mark with the same chloroform:methanol mixture.

The solution was then filtered through a 0.45 μm filter paper using a filtration kit. 1 ml of this solution was further diluted to 100 ml in a standard volumetric flask and made up to the mark with the dilution media. 20 μl, 9 μl and 3 μl of this solution were applied to a chromatographic plate for RIF, INH and PZA respectively and peak areas were recorded as reported in the calibration procedure.

The amounts of RIF, INH and PZA were then computed by external standard quantification. The results obtained are compared with that of the reported HPLC method [13].

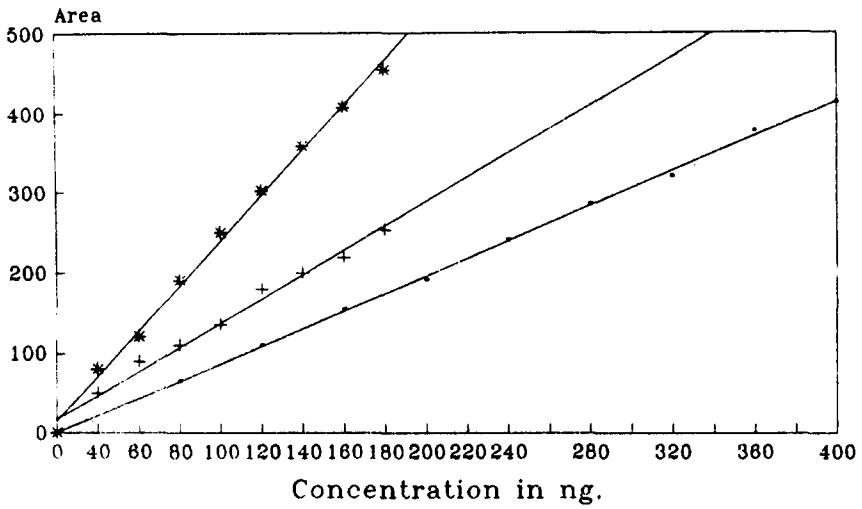

Fig. 1. Calibration curves for (●) RIF, (+) INH, (*) PZA.

2.8. Recovery studies

To study the accuracy, reproducibility and precision of the proposed method, recovery experiments were carried out. The recovery of the added standard was found at three different levels. The recovery experiment was repeated three times at each level. A plot of the amount of drug found by the proposed method (y axis) against the amount of standard added (x axis) was made. From the amount of drug found, the percentage recovery was calculated.

3. Results and discussion

The chromatogram developed in a mixture of ethyl acetate:ammonia:ethyl alcohol:cyclohexane, (20:9:4.5:5, v/v/v/v) gave good resolution of RIF, INH and PZA. There were three R_f values observed for standard RIF at 0.07, 0.10 and 0.20 which were confirmed to be 25-desacetyl rifampicin, rifampicin and rifampicin quinone respectively. The R_f values found for INH and PZA were 0.47 and 0.68 respectively. A typical densitogram showing the separations of RIF and its related substances, INH and PZA, is shown in Fig. 2. The spectra of all the tracks were recorded between 200 and 800 nm using a tungsten–deuterium lamp. A typical spectrum is shown in Fig. 3. A wavelength of 440 nm was used for quantification of RIF whereas a wavelength of 275 nm was used for quantification of INH and PZA.

A linear relationship was obtained for RIF, INH and PZA in the concentration ranges 120–400, 40–200 and 40-200 ng respectively. The calibration curves can be represented by the linear equations:

RIF: $y = 1.02393x - 10.6357$ $(r = 0.99493)$

INH: $y = 1.459935x - 3.61299$ $(r = 0.99705)$

PZA: $y = 2.519654x + 0.397403$

$(r = 0.999386)$

where y = area and x = amount of drug in nanograms. These equations were used for direct evaluation of the drugs.

In a replicate analysis ($n = 5$) of the tablet, the average contents of RIF, INH and PZA per tablet found by the proposed method were 122.89 mg, 80.36 mg and 252.02 mg with RSD 1.73%, 1.58% and 1.07% respectively. The amounts obtained by the reported HPLC method [13] were 121.80 mg, 80.56 mg and 251.56 mg with RSD 1.23%, 1.84% and 0.32% for RIF, INH and PZA respectively (Table 1). Thus the results obtained were comparable with the reported method.

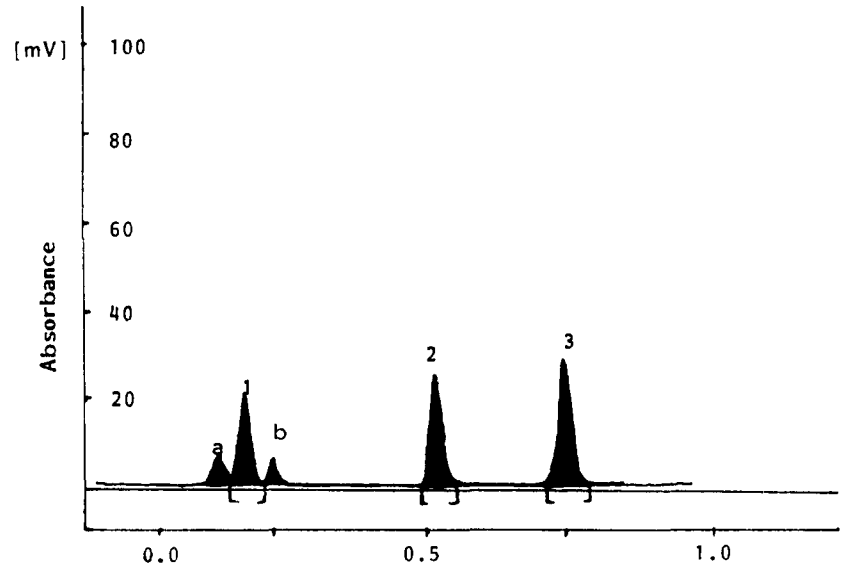

Fig. 2. Typical densitogram: (1) RIF: (a) 25-desacetyl rifampicin, (b) rifampicin quinone; (2) INH; (3) PZA.

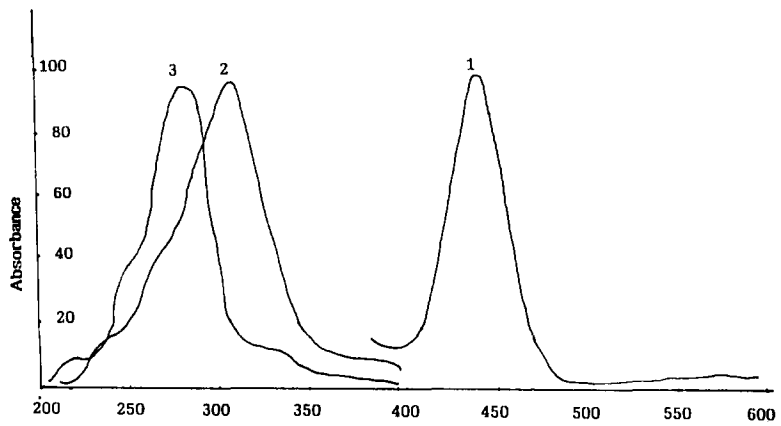

Fig. 3. Typical UV–Vis spectrum: (1) RIF; (2) INH; (3) PZA.

The B.P. methods [14] involve three different procedures for the assay of these three individual drugs which are applicable for single dosage forms. However, the proposed HPTLC method and the reported HPLC method [13] can be used for the simultaneous determination of these three drugs in combined dosage forms. In the HPLC method a tetrabutylammonium phosphoric acid buffer pH 3.0 is employed which shortens column life. The proposed HPTLC method is superior in this respect. It involves single step sample preparation and the three drugs can be effectively assayed by a single technique. Moreover, the related substances present in RIF do not interfere in the proposed method.

To study the accuracy, reproducibility and precision of the proposed method, recovery experiments were carried out. The recoveries obtained

Table 1
Results of HPTLC assay of tablet formulation (label claims: RIF, 120 mg per tablet; INH, 80 mg per tablet; PZA, 250 mg per tablet)

Amount found (mg per tablet) by HPTLC method			Amount found (mg per tablet) by HPLC method [13]		
RIF	INH	PZA	RIF	INH	PZA
123.98	78.39	255.36	121.00	79.90	251.34
125.63	79.92	253.89	123.56	78.45	250.32
119.89	81.53	248.30	119.78	82.13	251.21
122.56	80.63	251.20	122.90	81.78	252.54
122.39	81.36	251.39	121.76	80.56	251.89
Mean assay					
122.89	80.36	252.02	121.80	80.56	251.89
RSD (%)					
1.73	1.58	1.07	1.23	1.84	0.32

Table 2
Results of the recovery analysis (amounts in milligrams)

No. of levels	RIF (120.0 mg per tablet)		INH (80.0 mg per tablet)		PZA (250.0 mg per tablet)	
	Amount added	Amount recovered	Amount added	Amount recovered	Amount added	Amount recovered
1	00.00	121.56	00.00	81.35	00.00	251.39
2	30.00	153.30	20.00	98.56	20.00	271.10
3	40.00	161.10	30.00	109.89	30.00	280.32
4	50.00	170.32	40.00	122.45	40.00	290.10
Recovery	97.98		101.74		100.01	

were 97.98%, 101.74% and 100.01% for RIF, INH and PZA respectively (Table 2). The minimum detection limit was studied by reducing the concentrations of these drugs and processing as described above. The minimum detection limits were 20 ng for RIF and 10 ng for INH and PZA respectively.

4. Conclusion

The method described is sensitive, precise, rapid and involves single-step sample preparation. A large number of samples can be analysed within a short time, hence the method can be used for routine analysis in quality control and development laboratories.

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