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Short communication

Liquid chromatographic determination of isoniazid, pyrazinamide and rifampicin from pharmaceutical preparations and blood

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Abstract

Isoniazid (IN), pyrazinamide (Pz) and rifampicin (Rf) are separated on YMC-ODS column. IN was derivatized with 2-fluorene-carboxaldehyde (FA). The separation was achieved using ethanol–chloroform–acetonitrile water by isocratic elution and detected at 337 nm. The detection limits were 0.11 ng, 0.2 ng and 13 ng/injection (5 μl) for IN, Pz and Rf, respectively. The method of analysis was applied to the pharmaceutical preparations and in the blood samples of the patients suffering from tuberculosis after undergoing chemotherapy with IN, Pz and Rf. The amounts quantitated in blood showed 0.97 to 1.58 μg/ml IN, 3.44 to 4.09 μg/ml Pz and 1.98 to 3.5 μg/ml Rf with coefficient of variations 0.8–1.8%, 0.9–1.3% and 0.8–2.1%, respectively.

Keywords: Isoniazid; Pyrazinamide; Rifampicin

1. Introduction

Isoniazid (IN), rifampicin (Rf) and pyrazinamide (Pz) are all active bacterial antituberculosis drugs. Various analytical methods are reported for the determination of IN, Rf and Pz individually and in mixture [1–7]. The methods for the determination in mixture are mostly based on spectrophotometry or liquid chromatography.The spectrophotometric methods may involve their natural absorbance within the UV region or after derivatization with a suitable reagent, particularly for IN, to enhance the sensitivity [8–10]. Different reagents are used for the determination of IN separately and in the presence of Rf and Pz in formulation and in biological fluids using liquid chromatography [11–18]. The precolumn derivatizing reagents used for IN includes 4-hydroxybenzaldehyde, cinnamaldehyde and salicylaldehyde [19–23]. In the present work 2-fluorene-carboxaldehyde (FA) has been used as a precolumn derivatizing reagent for IN (Fig. 1), in the presence of Rf and Pz in pharmaceutical formulation and blood samples of pulmonary tuberculosis patients.

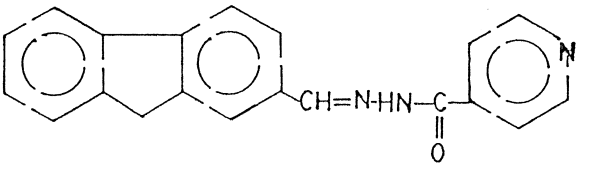

Fig. 1. Structural diagram of 2-fluorene-carboxaldehyde-isonicotinoyl-hydrazone.

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2. Experimental

Pure isoniazid (IN) (Nabi Qasim Pharmaceutical, Karachi, Pakistan), pyrazinamide (Pz) (Pacific Pharmaceutical, Lahore, Pakistan), rifampicin (Rf) (Abbot Lab. Pak., Karachi, Pakistan) and 2-fluorene-carboxaldehyde (FA) (Aldrich) were used. Spectrophotometric studies were carried out with a Hitachi 220 (Nissei Sangyo, Tokyo, Japan) spectrophotometer. IR spectrum in KBr was recorded on a Perkin-Elmer 1340 IR spectrophotometer. A Hitachi 655A liquid chromatograph with a variable wavelength UV monitor, Rheodyne 7125 injector and Hitachi D2500 Chromato-integrator were used. A YMC-ODS 5 μm, column (150×4.6 mm, I.D.) (YMC, Gibraltar Drive, Norris Plains, NJ, USA) was used. C, H and N elemental microanalysis of IN–FA was carried out by Elemental Microanalysis, Devon, UK. The solvents ethanol, chloroform and acetonitrite (E. Merck) were used. Freshly prepared double-distilled water was used for high-performance liquid chromatography (HPLC) studies.

Buffer solutions in the pH range 1 to 9 at an interval of 0.5 units were prepared from the following: Hydrochloric acid (0. 1 *M*), potassium chloride (1 *M*), acetic acid (1 *M*), sodium acetate (1 *M*), sodium bicarbonate (1 *M*) and sodium carbonate (saturated). The pH measurements were made with an Orion 420A pH meter.

2.1. Preparation of 2-fluorene-carboxaldehyde-isonicotinoyl-hydrazone (IN-FA)

Isoniazid (isonicotinoyl-hydrazine) (IN) (0.14 g) in ethanol (4 ml) was added to FA (0.2 g) in ethanol (20 ml) and hydrochloric acid (0.5 ml, 6 *M*). The contents were refluxed for 45 min and cooled overnight. The precipitate obtained was filtered and recrystallized from ethanol. Melting point 246°C, calculated for $C_{20}H_{15}N_3O$, expected % C=76.89, H=4.82, N=13.41; found % C=76.44, H=4.46, N=13.15. IR in KBr (/cm) 3490 (m), 3220 (m) for NH; 1670 (Vs), C=O, 1620 (s), 1570 (s), 1500 (m), 1460 (m), 1420 (m), C=N, C=C; 1310 (vs), 1230 (m), 1160 (m), 1080 (m), 1015 (w), 970 (s), 905 (m), 850 (s), 780 (s), 750 (s), 700 (s). UV methanol, λ-max nm (L/mole/cm) 337 (42 900), 210 (24 500).

2.2. HPLC determination

To solution (1–5 ml) containing IN (0–34.25 μg) or a mixture containing IN (0–34.27 μg), Pz (0–61.56 μg) and Rf (0–658.3 μg) in ethanol containing chloroform (5% v/v) was added FA (1 ml, 0.3% w/v in ethanol) and potassium chloride hydrochloric acid buffer (pH 2.5) (1 ml). The contents were heated on a water bath (70–80°C) for 10 min and the volume was adjusted to 10 ml with ethanol. The solution (5 μl) was injected on a YMC-ODS 5 μm, column (150×4.6 mm, I.D.) and elution was carried out with ethanol–water–chloroform–acetonitrile (55:40:4:1, v:v:v:v) with a flow-rate of 1.4 ml/min. The detection was carried out at 337 nm.

2.3. Solvent extraction for isoniazid and rifampicin

An aqueous ethanolic solution of IN (1 ml) containing (0–13.0 μg) and ethanolic solution of Rf containing (5%) chloroform (1 ml) (0–160 μg) was transferred to a well-stoppered test tube (Quickfit) and to this were added FA (1 ml, 0.3% w/v in ethanol) and potassium chloride hydrochloric acid buffer pH 2.5 (1 ml). The contents were heated on a water bath (70°C) for 10 min. To the cooled solution water and chloroform (2 ml of each) was added and then shaken on a mechanical shaker for 5 min. An aliquot of the organic layer was transferred to a screw-cap sample vial and a 5 μl solution was injected on a YMC-ODS column and elution was carried out according to Section 2.2.

2.4. Analysis of isoniazid, pyrazinamide and rifampicin in pharmaceutical preparations

Six tablets of B.P. Rimactazid 450, Rifinah 450, Myrin or Myrin-P were crushed and ground to fine powder, samples 98.12 mg from Rimactazid 450, 92.29 mg Myrin-P, 166.61 mg Rifinah or 68.33 mg Myrin tablets were dissolved in ethanol containing chloroform (5% v/v). The dissolution was carried out three times with a portion of 20 ml each by warming on a water bath (70°C). The solution was filtered through Whatman filter paper 42 before adjusting the volume to 100 ml with ethanol. The solution (1 ml) was taken and processed as in Section 2.2. The amounts of IN, Pz and Rf were

evaluated from a calibration curve prepared from known amounts of each compound.

2.5. Analysis of isoniazid, pyrazinamide and rifampicin from blood samples

Blood samples (3 ml) of the various patients suffering from pulmonary tuberculosis were collected 4–5 h after administration of the drugs. The blood was kept at room temperature (30°C) for about 2–3 h to coagulate. The serum was separated after centrifugation for 15 min at 3000 *g*. The serum (1.5 ml) was mixed with FA (2 ml, 0.3% w/v in ethanol), trichloroacetic acid (1 ml, 10%, w/v in ethanol) and hydrochloric acid–potassium chloride buffer pH 2.5 (1 ml). The mixture was heated on a water bath (70°C) for 10 min, cooled to room temperature and then ethanol (1 ml) and chloroform (0.5 ml) were added. The final volume was adjusted to 7 ml. The contents were centrifuged again at 3000 *g* for 15 min. A clear supernatant layer was separated and 5 μl of the solution was injected on a YMC-ODS column (150×4.6 mm, I.D.) and elution was performed as in Section 2.2.

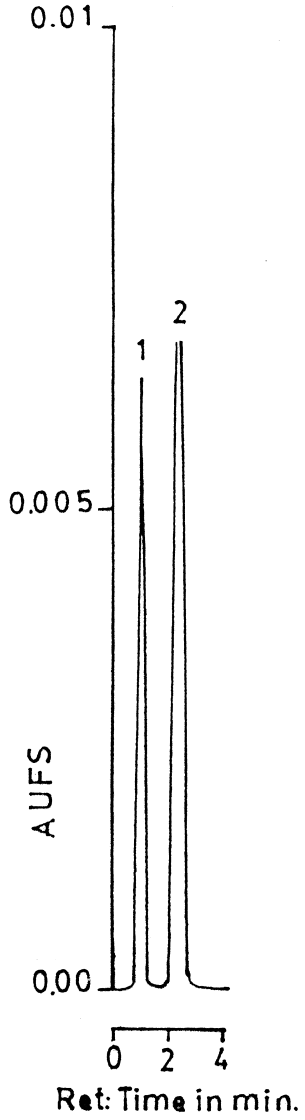

Fig. 2. HPLC separation of (1) IN-FA (2) FA, Column YMC-ODS, 5 μm (150×4.6 mm, I.D.), elution with methanol:water (65:35, v:v) at a flow-rate 1.4 ml/min with the detection UV at 337 nm.

2.6. Recovery of isoniazid from pharmaceutical preparation and blood samples

A sample of 98.12 mg was taken from six well-ground tablets of Rimactazid 450 and was processed as in Section 2.4. Another sample of 98.12 mg was taken and spiked with 13.7 and 27.4 μg of IN. The sample was again processed as in Section 2.4.

Blood samples taken from normal volunteers (5 ml) was processed as in Section 2.5 and to another sample (5 ml) was added IN 1.37 μg and proceeded again as in Section 2.5. The increase in the response was used to calculate the % recovery. Quantitation was made using external calibration.

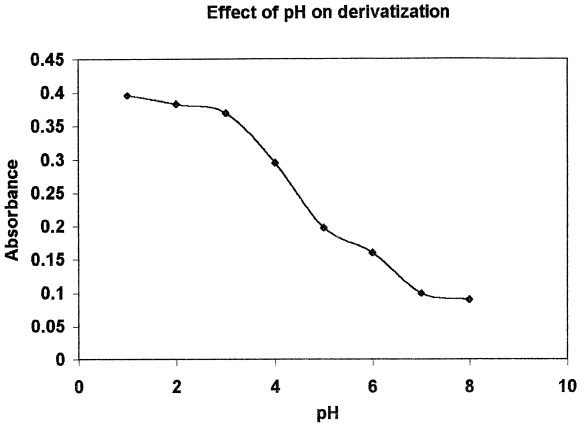

Fig. 3. Effect of pH on the derivatization of IN-FA. The absorbances were measured at 337 nm.

Table 1
Variation in the absorbance of Rf and Pz using hydrochloric acid–potassium chloride buffer at pH 2.5 over 24 h

Time (h)	Pz	Rf
0	0.487	0.404
2	0.487	0.403
4	0.494	0.401
12	0.495	0.398
24	0.454	0.391

The absorbances were measured at 337 nm.

3. Results and discussion

The IN was reacted with FA in slightly acidic medium in 1:1 molar ratio to form IN-FA. The elemental micro analysis (C, H and N) agreed to the structure assigned (Fig. 1). The IR spectrum recorded in KBr also indicated bands expected from the structure. The UV absorption spectra in methanol indicated two bands at 210 nm and 337 nm with molar absorptivity of 2.4×10^4 and 3×10^4 L/mole/

Table 2
Analysis of isoniazid, pyrazinamide and rifampicin in pharmaceutical preparations

S No.	Name of pharmaceutical preparations	Compounds present	Amount reported mg/tablet	Amount found by the HPLC mg/tablet (SD)	% Relative deviation
1	Myrene-P[a]	Isoniazid	60	59.0 (0.25)	1.7
		Pyrazinamide	300	287.8 (3.36)	4.0
		Rifampicin	120	119.0 (0.36)	0.8
2	Myrene[a]	Isoniazid	75	73.8 (0.46)	1.6
		Rifampicin	150	152.0 (1.96)	1.3
		Ethanbutol	300	–	–
3	Rimactazid 450[b]	Isoniazid	300	289.9 (1.75)	3.8
		Rifampicin	450	428.0 (3.82)	4.9
4	Rifinah 450[c]	Isoniazid	300	288.5 (3.15)	3.8
		Rifampicin	450	430.0 (3.90)	4.4

[a] Lederle Labs. Div. Cyamid, Karachi, Pakistan.
[b] Ciba-Geigy, Karachi, Pakistan.
[c] Pacific Pharma, Lahore, Pakistan.

Table 3
HPLC analysis of isoniazid, pyrazinamide and rifampicin in blood samples of patients suffering from pulmonary tuberculosis

S No.	Name of preparation	Name of drugs present	Amount of each drug reported (mg/tablet)	Amount of each drug taken by the patient in a single dose (mg/day)	Amount of each drug found by HPLC μg/ml (C.V.%) Patient no.						
					1	2	3	4	5	6	7
1	Myrene-P	Isoniazid	60	180	0.82 (1.2)	0.90 (0.83)	0.79 (1.8)	–	0.78 (1.31)	0.86 (1.20)	0.81 (1.65)
		Pyrazinamide	300	900	3.44 (1.1)	4.09 (1.30)	3.37 (0.9)	–	3.80 (1.85)	3.85 (1.46)	0.88 (1.27)
		Rifampicin	120	360	2.05 (0.8)	2.10 (1.70)	1.98 (1.1)	–	1.99 (1.40)	2.07 (1.20	1.79 (1.45)
2	Rifampin	Rifampicin	450	450	–	–	–	3.50 (1.7)	–	–	–
3	Mayambutol-INH	Isoniazid	100	300	–	–	–	1.58 (1.4)	–	–	–
		Ethambutol	300	900	–	–	–	–	–	–	–

cm which represented a considerable enhancement in natural absorbance of IN at 263 nm in comparison to its normal molar absorptivity value of 4433 L/mole/cm.

For the sensitive determination of IN in pharmaceutical preparations and serum, precolumn derivatization followed by elution on YMC-ODS column was examined. IN-FA was easily eluted as a symmetrical peak with methanol:water (65:35, v:v) and separated completely from the derivatizing reagent FA (Fig. 2).

For the quantitative determination of IN the derivatization conditions were optimized by measuring average peak height ($n=3$). The effects of pH, concentration of derivatizing reagent, reaction time (70–75°C) and solvent, on the derivatization were examined. The final volume was adjusted to 10 ml. A constant amount (5 μl) was injected for HPLC elution and the conditions, which gave maximum responses, were considered as optimal. The absorbance of the solutions at 337 nm was also recorded and any change in absorbance with pH, concentration of derivatizing reagent and heating time (70–75°C) was noted. Volumes of 0.1 to 0.5 ml of hydrochloric acid (6 *M*) were added at intervals of 0.1 ml and buffer solutions (1 ml) in a pH range from 1 to 4.5 at intervals of 0.5 were added.

It was observed that a decrease in the response of IN derivative occurred above pH 3. However a constant response was observed by using buffer solution of pH 2.5 and below (Fig. 3). The effect of pH and acid concentration on solution stability of Rf and Pz was also examined spectrophotometrically. A decrease in absorbance of Rf at 337 nm with respect to time was observed with an increase in the concentration of hydrochloric acid (0.1 to 0.5 ml of 6 *M* HCl). However by using the buffer solution of pH 2.5, the decrease in absorbance of Rf in 4 h was found to be within 5% (Table 1). Using the conditions, change in absorbance of Pz with time was investigated. Any change in absorbance of Pz at pH 2.5 was not observed (Table 1). Reagent concentration was varied between 1 to 4 ml of 0.3% in ethanol and addition of 2 ml proved to be satisfactory. Reaction times were varied between 5 to 30 min at intervals of 5 min. A similar response was obtained after heating for 5 min and therefore heating time of 10 min was selected. Finally KCl–HCl buffer pH 2.5 (1 ml), 2 ml of (0.3%) of reagent FA solution, heating time 10 min at 70°C, solvent ethanol containing 5% chloroform and a final volume of solution was selected to be 10 ml.

IN in dosage form is given individually and in combination with Pz and Rf. The separation of IN, Pz and Rf showed that Rf was long retained and

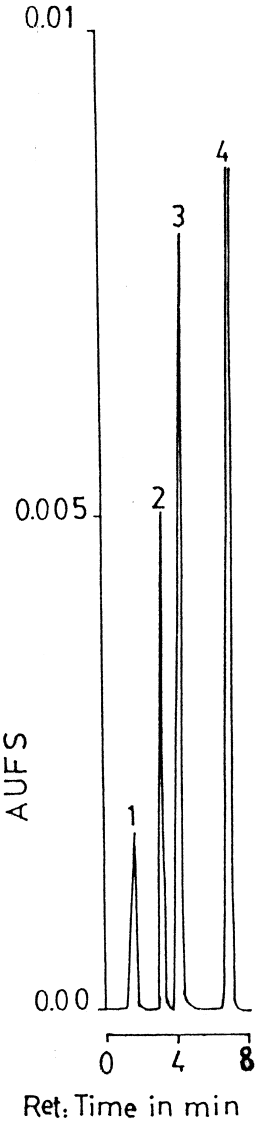

Fig. 4. HPLC separation of (1) pyrazinamide (2) rifampicin (3) IN-FA (4) FA, Column YMC-ODS (150×4.6 mm, I.D.), elution with ethanol–water–chloroform–acetonitrile (55:40:4:1, v:v:v:v) at a flow-rate of 1.4 ml/min with detection UV at 337 nm.

gave a broader peak when eluted with aqueous–methanol or aqueous–ethanol. However addition of acetonitrile and chloroform in the aqueous ethanol gave satisfactory results with reasonable peak shape. The addition of ethanol and chloroform effected the elution and capacity factor k' of the compounds Pz, Rf, IN-FA and FA which gave k' 0.663, 3.791, 5.302 and 9.488 when eluted with ethanol–water–chloroform–acetonitrile (55:40:3:2, v:v:v:v). The values of k' increased to 0.826, 3.186, 6.512 and 11.419 when the amount of ethanol was decreased by 5%. The values of k' decreased to 0.512, 2.640, 3.942 and 7.512, respectively, when the amount of chloroform was increased from 3 to 4% with retention time within 8 min. (Fig. 4). The resolution factor (*Rs*) between two adjacent peaks was observed to be greater than 1.5.

Linear calibrations were obtained by plotting average peak height ($n=3$) against concentration with IN 0.658–3.427 μg/ml, Pz 1.231–6.156 μg/ml and Rf 16.459–65.831 μg/ml at 337 nm with coefficient of correlation (*r*) 0.999, 0.996 and 0.9989, respectively, from ($n=5$) points calibrations. The detection limits measured as the response, three times the background noise were 21.92 ng/ml, 39.4 ng/ml and 263 ng/ml for IN, Pz and Rf corresponding to 0.11 ng, 0.2 ng and 13 ng per injection (5 μl), respectively.

To separate from aqueous ethanolic solution, solvent extraction procedure in chloroform was examined. It was observed that average transfer ($n=4$) of IN and Rf was 96% and 98% with coefficient of variation (C.V.) 1.2% and 0.9%. However the transfer of pyrazinamide was not quantitative. The pH from 1 to 9 did not effect the extraction of Pz. Therefore for the simultaneous determination of IN, Pz and Rf aqueous ethanolic solution containing chloroform (5%) was used.

When the additives glucose, lactose, talcum, magnesium stearate and gum acacia were added 10 times the concentration of IN (13.7 μg/ml), the quantitative determination of IN was not effected.

The pharmaceutical preparations Myrin, Myrin-P, Rimactazid 450 and Rifinah 450 were analyzed for the contents of IN, Pz and Rf and the results observed (Table 2) indicate C.V. within 0.3–1.2%. The average results ($n=3$) also indicate close correlation with the expected values with % relative deviation within 0.8–4.9. The percentage recovery of IN from Rimactazid 450 tablets was checked by spiking with known amounts of IN 13.7 and 27.4 μg, and the increase in the peak height was used to calculate the amount of isoniazid recovered. The amount of isoniazid extracted from Rimactazid 450 tablets was calculated at 95.5% with C.V. 0.97%.

The method was used for the determination of IN, Pz and Rf in the serum of patients suffering from

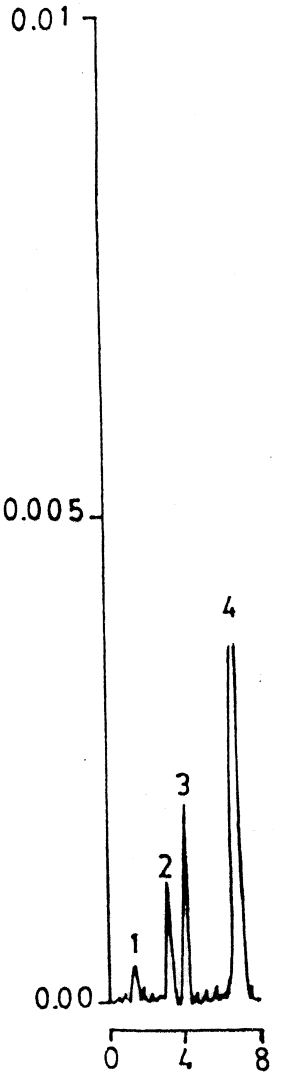

Fig. 5. HPLC determination of (1) pyrazinamide 2.05 μg/ml (2) rifampicin 3.44 μg/ml (3) isoniazid 0.82 μg/ml from blood samples of a patient suffering from pulmonary tuberculosis after chemotherapy with Myrin-P. Conditions as in Fig. 4.

pulmonary tuberculosis (Fig. 5). The gain of the chromato-integrator was increased by decreasing the attenuation. New calibration curves were constructed in the range of 0.14–0.55 μg/ml IN, 2.46–14.77 μg/ml Pz and 1.6–6.5 μg/ml Rf, followed by the analysis of the drugs on the same day. The amount of IN found was within 0.97–1.58 μg/ml with C.V. 0.83–1.8%, Pz 3.44–4.09 μg/ml with C.V. 0.9–1.3% and Rf 1.98–3.5 μg/ml with C.V. 0.8–2.1% in blood samples (Table 3).

Finally % recovery of IN in blood samples from healthy volunteers who had not used any medicine at least for 1 week was studied. Known amounts of IN were added to the blood samples and then processed as in Section 2.5. The average peak height ($n=3$) showed % recovery of IN as 93.8% with C.V. 3.68%.

4. Conclusion

A HPLC method is proposed for the quantitation of IN, together with Pz and Rf. IN is precolumn derivatized with FA. Adequate separation is obtained between IN, Pz and Rf for their determination at μg/ml in pharmaceutical preparations and at sub μg/ml in blood. The common additives present together with the drugs do not affect the determination. The method indicates viable application for their quantitation from blood.

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