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Capillary Gas Chromatographic Determination of Isoniazid in Pharmaceutical Preparations and Blood by Precolumn Derivatization with Trifluoroacetylacetone

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ABSTRACT

Antituberculosis drug isoniazid (INH) and hydrazine (HZ) have been determined by capillary column gas chromatography after precolumn derivatization with trifluoroacetylacetone (FAA). Phenylhydrazine (PHZ) when present together with INH and HZ also separated completely from the column HP-5 (30 mm × 0.32 mm I.D) connected with flame ionization detection (FID), and thus was used as an internal standard. The linear calibration ranges for INH and HZ were determined to be 2.5-25 μg/mL and 2.5-21.2 μg/mL respectively, with detection limit (S/N = 3) corresponding 62.5 pg reaching to the detector. The method was applied for the determination of INH and HZ from pharmaceutical preparations. The method was also used for determination of INH from blood of tuberculosis patient. The amount of INH found in the blood serum of tuberculosis patients on chemotherapy with INH based drugs was within 0.82-4.8 μg/mL with relative standard deviation (RSD) of 2-5.8%. The recovery percentage of INH from blood samples was found 98% with RSD 2.5%.

Key words: Isoniazid, Hydrazine, Trifluoroacetylacetone, Capillary gas chromatographic determination

INTRODUCTION

INH (isonicotinoylhydrazine) is the most potent and selective tuberculostatic antibacterial agent in the therapy of tuberculosis[1]. It inhibits the growth of *Tubercle bacillus in vitro* in concentration less than 1 μg/mL[2]. It is also used as a propylactic agent for persons constantly exposed to tubercular patients. INH gains access to all organs and all body fluids including cerebrospinal fluids and this character renders the drug of special value in treating tuberculosis meningitis and other extra pulmonary forms of the disease. When used alone, it is at least equal to streptomycin in the therapy of tuberculosis. It is believed to affect lipids, nucleic acids, and glycolysis or mycolic acid biosynthesis[3]. HZ is toxic substance and may be present in INH tablets as the decomposition product[4].

Various analytical techniques such as titrimetry[5], spectrophotometry[6-8], spectro-fluorimetry[9], atomic absorption[10], chemiluminescence[11], electroanalytical techniques[12-13], kinetic determination[14], flow injection[14,16], thin layer chromatography[17-18], capillary electrophoresis[19-20] and liquid chromatography[21-24] have been used for the quantitation of INH in pharmaceutical preparations and biological samples. The liquid chromatography with UV detection is carried out either by measuring the natural absorbance of INH at 263 nm or by precolumn derivatization with a suitable derivatizing reagent[25-27]. The determination limits for INH has been reported within 0.5-8.0 μg/mL[24]. The gas chromatography (GC) of INH was carried out after derivatization with trifluoroacetic anhydride or bis(trimethylsilyl)trifluoroac etamide and quantitation was carried by mass spectrometry[28-29]. The use of acid anhydride affected the performance of GC column. β-diketone reacted with amino compounds to form Schiff bases and addition of carbon chain could enhance FID sensitivity. FAA has been used for GC determination of putresine and cadverine from biological fluids[30].

The present work examines GC determination of INH and HZ after derivatization with FAA using PHZ as an internal standard. The volatile derivatives are easy to elute and separate from capillary GC column with required sensitivity for the desired application for the analysis of biological fluids.

MATERIALS AND METHODS

I. *Materials and Methods*

All the chemicals used were of reagent or pharmaceutical grade. Freshly prepared double distilled water was used throughout the study.

Pure INH was obtained from Nabi Qasim Pharmaceuticals, Karachi, Pakistan. HZ (24%), PHZ, FAA and methanol were purchased from E. Merck, Darmstadt, Germany. The percentage of HZ in HZ solution was determined by the titrimetry. Hydrochloric acid (37%), potassium chloride, acetic acid, sodium acetate, ammonium acetate, sodium bicarbonate, sodium carbonate,

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ammonium chloride were also obtained from E-Merck Germany. Buffer solutions in the pH range 1-10 at unit interval were prepared from hydrochloric acid (0.1 M) and potassium chloride (1 M) (pH 1 &2), acetic acid (1 M) and sodium acetate (1 M) (pH 3 to 6); ammonium acetate (pH 7), sodium bicarbonate (1 M) and sodium carbonate (saturated) (pH 8 & 9), ammonium chloride (1 M) and ammonia (1 M) (pH 10).

Freshly prepared solution of INH was used by dissolving 10.95 mg INH in methanol: water (1:1 v/v) and final volume was adjusted to 10 mL. FAA (1% v/v) was prepared in 100 mL of methanol: water (1:1 v/v).

The spectrophotometric studies were carried out on double beam Hitachi 220 (Hitachi (Pvt) Ltd. Tokyo, Japan) spectrophotometer with dual 1 cm cells. Gas chromatographic studies were carried out on Agilent model 6890 Net work GC system (Agilent Technologies Inc. USA) coupled with FID, split/splitless injector operated in split mode, hydrogen generator (Parker Balston, Analytical Gas system H2-90, Parker Hannifin, Havorhill, MA, USA) and pure nitrogen (British Oxygen Company, Karachi). The computer with Chemstation software controlled the gas chromatograph, and HP LaserJet 1300 printer was used throughout the study. Capillary column HP-5 (30 mm × 0.32 mm I.D) with film thickness of 0.25 μm (J & W scientific GC columns, USA) was used throughout the study. The Orion model 420A pH meter (Orion Research Inc, Boston, USA) with glass electrode with combined reference electrode was used for pH measurements.

II.*Gas Chromatographic Determination*

The solution (1 mL) containing INH(1-20 μg), HZ(1-17 μg) and PHZ(5 μg) was added 1 mL of potassium chloride-hydrochloric acid buffer pH 2, 1 mL of (1% v/v) FAA and heated at 75°C for 15 min. The solution was cooled at room temperature and were added 1 mL of chloroform. The contents were mixed well and layers were allowed to separate. Exactly 0.5 mL of chloroform was pipetted out and transferred to screw capped vial. The solvent was evaporated under nitrogen gas and re-dissolved in 0.2 mL of methanol. The solution (1 μL) was injected on capillary GC column HP-5 at a column temperature of 100°C with heating rate 30°C up to 280°C. The total run time was 7 min and nitrogen flow rate was 1 mL/min. The split ratio was 20:1. The injection port and detector temperatures were fixed at 200°C and 300°C respectively. Hydrogen and nitrogen flow rates were fixed at 40 and 45 mL/min respectively for FID detection.

III. *Determination of Isoniazid in Pharmaceutical Preparations*

Ten tablets of each Remactal, Remister (Novartis Pharma, Pak. Ltd.) and Myrin P (Leaderle Laboratories Division, Cyanamid, Pak. Ltd., Karachi) were powdered and 53.89 mg Remactal, 53.90 mg Remistar and 57.12 mg Myrin were dissolved in each portions(6 mL) of methanol: water (1:1) respectively. The solution was filtered and final volume was adjusted to 50 mL with methanol:water (1:1 v/v) respectively. The solution (1 mL) was transferred to screw-capped vial and 1 mL of chloroform was added. The contents were mixed well and the layers were allowed to separate. The aqueous layer was collected. The analytical GC procedure II. was then followed after the addition of 5 μg of PHZ as internal standard.

Ten tablets of INH (Unexo Lab. Ltd, Lahore) were thoroughly ground and the powder (51.0 mg) was dissolved in methanol:water (1:1 v/v). The solution was filtered and the final volume was adjusted to 100 mL. 1 mL was taken and the analytical procedure II. was followed after the addition of 5 μg of PHZ as internal standard.

5 mL of well-mixed INH syrup was dissolved in methanol:water (1:1 v/v), the solution was filtered and volume was adjusted to 100 mL. The solution was diluted 50 times and 1 mL was processed as analytical procedure II.. The amounts of INH in pharmaceutical preparations were evaluated from ratio of the peaks of the analyte and internal standard and external calibration curve.

IV. *Determination of Hydrazine from Isoniazid Formulations*

0.5 mL of well-mixed INH syrup containing (5 mg) INH was processed as analytical procedure II.. Ten tablets of isoniazed were thoroughly ground the powder (1 g, containing 545.5 mg INH) was dissolved in methanol: water (1:1 v/v) and the solution was filtered and adjusted to 25 mL. The solution 1 mL was taken and the analytical procedure II. was followed. The signals corresponding to HZ and PHZ were recorded.

V. *Determination of Isoniazid from Blood Samples*

Blood sample (5 mL) of various patients suffering from pulmonary tuberculosis was collected 2-4 hr after the administration of the drug. The samples were collected from the patients by vinipuncture with disposable syringe from Tuberculosis Hospital, Kotri. The blood samples, was incubated for 1 hr at (30°C) and centrifuged at 3000 g for 10 min. The supertenant layer was collected and was added methanol twice in the volume. The mixed contents were centrifuged at 3000 g for 10 min. The supertnant was transferred into a vial and 2 mL of chloroform was added. The contents were mixed well and layers were allowed to separate. The upper layer was pipetted out into vial and PHZ 5 μg was added. The derivatization procedure II. then was followed.

VI. *Recovery of Isoniazid from Blood Samples*

Blood samples (5 mL) collected from a healthy volunteer was processed as above and blood serum was added INH (6 μg) and processed as above using FAA as derivatizing reagent. The amount of INH was calculated from external calibration curve and internal standard.

Journal of Food and Drug Analysis, Vol. 14, No. 4, 2006

RESULTS AND DISCUSSION

INH and HZ were conjugated easily with FAA to form trifluoroacetylacetone-isonicotinyl hydrazone and bis(trifluoroacetylacetone) hydrazone (Fiure 1). The presence of trifluoromethyl group in FAA could enhance the volatility and thermal stability of the conjugated. Therefore, FAA was selected as the derivatizing reagent for the selective and sensitive determination of INH and HZ by capillary column GC. Precolumn derivatization was carried out, and the elution was executed from the capillary column HP-5 at an initial column temperature 100°C with heating rate 30°C up to 280°C. The run time was 7 min. Nitrogen flow rate was 1 mL/min. Each of the derivatives INH and HZ gave a single peak with retention time of 6.5 min and 3.9 min respectively. The derivatives were separated completely from the derivatizing reagent.

The derivatization conditions were optimized for the quantitative determination of INH and HZ by measuring average peak height (n = 3). The effects of pH, the concentration of derivatizing reagent and reaction time at 70-80°C were examined. A solution of 1 µL was injected with split ratio 20:1 and the condition, which gave maximum response, was considered optimum.

The pH was varied between 1-10 at unit interval and was observed that derivatization occurred in acidic media (pH 1-3) and a decrease into the response was observed above pH 3. The optimal response was obtained at pH 2 (Figure 2). The derivatizing reagent concentration was varied between 1-3 mL (1% v/v) at an internal of 0.5 mL. The average peak height (n = 3) was plotted against the amount of reagent solution added and a similar response was obtained at the amount of 1 mL and above, thus 1 mL was used. Heating time was varied between 5 to 25 min. at 75°C at an interval of 5 min and same average peak height (n = 3) was obtained after heating time of 10 to 20 min and 15 min was selected.

Under the same conditions the hydrazino compound: PHZ was also examined. The compound formed derivative with FAA eluted separately (Figure 3) and did not affect the determination of INH and HZ. PHZ was therefore used as an internal standard.

At the optimized conditions, linear calibration curves for the simultaneous determination of INH and HZ were obtained by measuring average peak height (n = 3) with 2.5-25 µg/mL INH and 2.5-21.2 µg/mL HZ with co-efficient of determination (r^2) 0.9806 and 0.9827 respectively using (n = 5) points calibration. The detection limits measured as signal to noise ratio 3:1 were obtained with 1.25 µg/mL for INH and HZ corresponding to 62.5 pg reaching to the detector with split ratio 20:1.

Common additives glucose, magnesium stearate, gum acacia, talcum, methylparaben, lactose and starch, when added twice the concentration of INH and HZ, did not interfere. The method was applied for the determination of INH from pharmaceutical preparations: Ramactal, Remister, Myrin, INH tablets and INH syrup. The hydrazine was determined from INH tablets and INH syrup. The GC results are summarized in Table 1 and 2. The INH tablets and INH syrup were analyzed after dissolution of INH in methanol-water (1:1 v/v) (Figure 4). The tablets Remactal, Remster and Myrin contained rifampicin, pyrazinamide and ethambutal together with INH. INH after derivatization with FAA was extracted in chloroform and pyrazinamide and ethambutol remained in aqueous phase and did not interfere the determination of INH. Rifampicin is extracted in chloroform together with

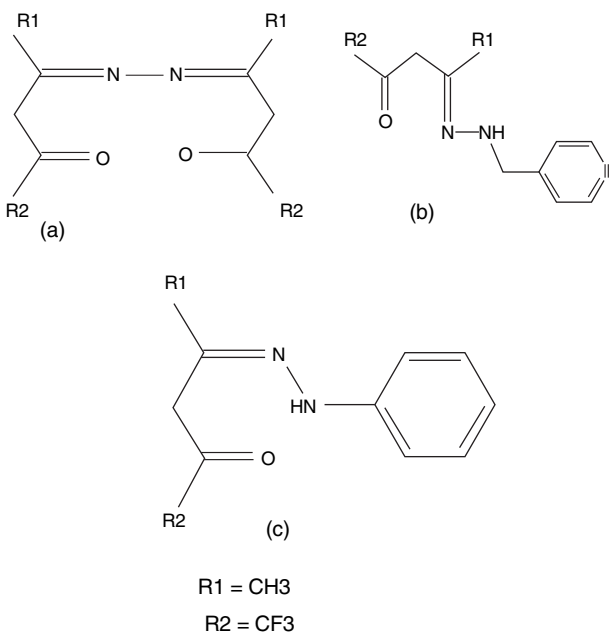

Figure 1. Structure diagrams of derivatives with trifluoroacetylacetone (FAA) (a) hydrazine (HZ) (b) isoniazid (INH) (c) phenylhydrazine (PHZ).

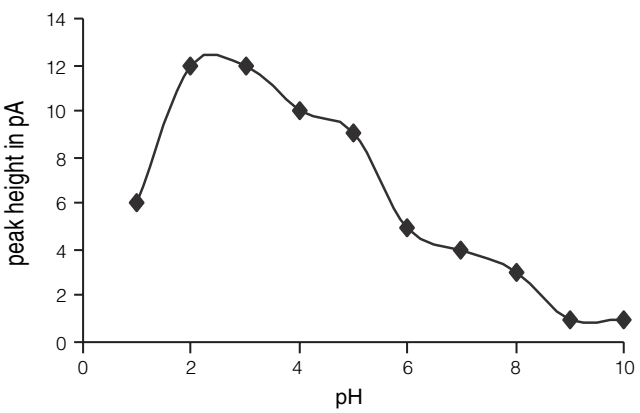

Figure 2. Effect of pH on the GC elution of FAA-INH derivative. GC conditions: The column HP-5 (30 m × 0.32 mm) with film thickness 0.25 µm at an initial column temperature 100°C with heating rate 30°C up to 280°C. The run time was 7 min. Nitrogen flow rate was 1 mL/min and split ratio 20:1. Injection port and detector temperatures were 200°C and 300°C. Nitrogen make up flow rate was 45 mL/min. FID air and hydrogen flow rates were 450 mL and 40 mL respectively.

Table 1. The determination of isoniazid from pharmaceutical preparations by using FAA as derivatizing reagent

S.NO.	Name of tablet	Compounds Present	Amount of compounds labeled (mg/tablet)	Amount of Isoniazid found in mg (RSD%)	% Relative deviation
1	Remactal	Isoniazid Rifampicin	150 300	147(8.5) –	2.0
2	Remistar	Isoniazid Rifampicin Pyrazinamide Ethambutanol	75 120 400 275	70(7.5) – – –	6.7
3	Myrin	Isoniazid Rifampicin Pyrazinamide Ethambutanol	60 150 400 275	58 (4.0) – – –	3.0
4	Isoniazid Syrup	Isoniazid	50 mg/5 mL	48mg/5mL (5.6)	4.0
5	Isoniazid Tablet	Isoniazid	100	101 (4.5)	1

Table 2. Determination of hydrazine from pharmaceutical preparations by using FAA as derivatizing reagent

Sample	Isoniazid syrup	Isoniazid tablet
Amount of INH labeled in mg	1 mL contains10mg of INH	1000 mg of tablets contain 454.5 mg INH
Amount of HZ found in µg in INH formulations (RSD%)	1.0 µg/5 mg of INH syrup (3.2)	5 µg HZ/18.18 mg of INH in tablets (2.5)

Table 3. Analysis of isoniazid from blood samples of T.B meningitisafter chemotherapy with 300 mg of isoniazid using FAA as derivatizing reagent

Patients No.	Gender	Age	Amount of isoniazid found µg/mL blood (RSD %) (n = 3)	
1	Male	38	4.8	(3.2)
2	Male	24	2.5	(5.2)
3	Male	30	2.0	(3.9)
4	Male	40	0.82	(2.0)
5	Female	42	1.0	(5.8)

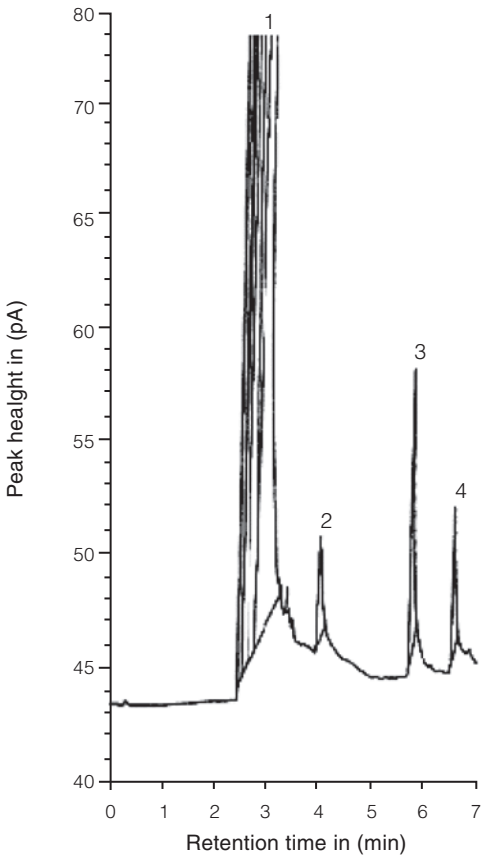

Figure 3. GC separation of (1) solvent & FAA (2) HZ (3) PHZ (4) INH as derivative of FAA. GC conditions as Figure 2.

INH derivative, but did not elute as symmetrical peak, and disturbed the base line due to on column decomposition. However rifampicin separated completely, when extracted in chloroform prior to derivatization of INH in aqueous-methanolic solution and did not affect the determination of INH. The relative percentage deviations were found 1 to 6.7% from the values labeled by the manufacturer with relative standard deviation within 4.0-8.5% (Table 1) The RSD is slightly on higher side because of the presence of multi- component system. HZ was checked for its presence in INH syrup and INH tablets. INH syrup containing 5 mg of INH and INH tablets containing 18.18 mg INH were analyzed for HZ and PHZ was added as internal standard. The signals corresponding to HZ, and PHZ were recorded. The Table 2 indicated the presence of 1.0 µg HZ/5 mg INH in INH syrup and 5 µg HZ/18.18

Journal of Food and Drug Analysis, Vol. 14, No. 4, 2006

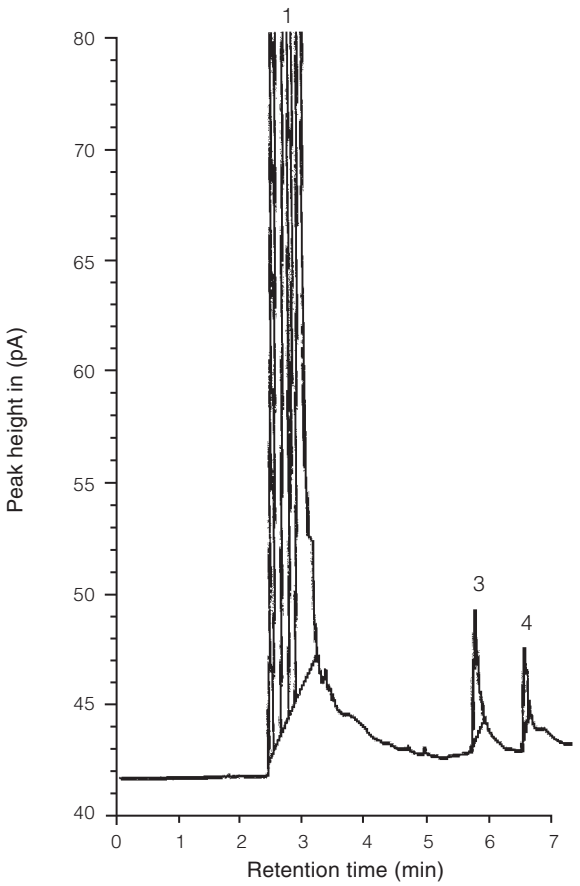

Figure 4. GC response for INH from INH syrup. GC conditions as Figure 2.

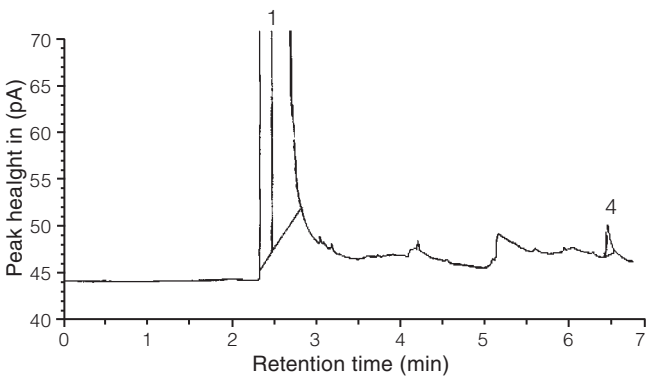

Figure 5. GC response of INH from the blood sample of healthy volunteer after spiking with INH 6 μg. GC conditions as Figure 2.

mg INH in INH tablets. Blood samples of patients suffering from tuberculosis were collected after 2-4 hr of chemotherapy with a single dose of 300 mg of INH. The blood sample after deproteinization was extracted with chloroform to remove the presence of any rifampicin. The INH after derivatization with FAA was determined. The amount of INH in the blood was observed within 0.82-4.8 μg/mL with RSD 2.0-5.8% (Table 3). The recovery percentage of INH from the blood was examined by spiking the serum of healthy volunteer with INH and the amount of INH recovered was evaluated from calibration curve. The amount of recovery was 98% with RSD 2.5%. (Figure 5)

CONCLUSIONS

Simple GC procedure has been developed for the determination of INH from pharmaceutical preparations and blood samples after precolumn derivatization with FAA. HZ was also determined from INH formulations and PHZ was as internal standard. The detection limits were obtained at 62.5 pg reaching to the detector. The analysis of pharmaceutical preparations and blood samples after chemotherapy were observed with RSD 3.2-5.8%.

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