CHROMATOGRAPHIC STUDIES ON ISONICOTINIC ACID HYDRAZIDE AND ITS METABOLIC DERIVATIVES

IV. NEW TECHNIQUES OF ISOLATION AND IDENTIFICATION

R. C. R. BARRETO AND S. O. SABINO

Department of Biochemistry, Institute of Phthisiology and Pneumology, University of Brazil, Rio de Janeiro (Brazil)*

(Received July 2nd, 1963)

INTRODUCTION

Various techniques for the isolation of isonicotinic acid hydrazide (INH) and its metabolic derivatives from samples of biological material have already been developed in our laboratory, such as centrifugal ultrafiltration combined with descending paper chromatography[1], paper electrophoresis[2,3] and direct chromatographic separation of the metabolites from the wet sample[4].

The application of paper electrophoresis is limited to the isolation of acid metabolites and, as in the case of direct chromatographic separation from wet samples, can only be used when large amounts of the metabolic derivatives are present. Centrifugal ultrafiltration combined with descending paper chromatography allows the concentration of the metabolic derivatives but is, however, a laborious and time-consuming technique.

We have now developed a technique for the isolation of INH-derivatives from biological samples, such as serum and plasma, based on its dehydration in columns of anhydrous sodium sulphate and subsequent elution of the said derivatives with a mixture of chloroform and diethylamine. Fairly large samples can thus be used, the eluted material being easily concentrated.

The good results obtained with diethylamine for the elution of INH-derivatives led us to study this reagent in solvent mixtures for the paper chromatography of the above compounds. Furthermore, new reagents have been investigated for the localization and identification of INH-derivatives on paper chromatograms.

MATERIALS AND METHODS

Isolation of INH-derivatives by column chromatography

Various materials were tested for the chromatographic isolation of INH-derivatives, such as silica gel, anhydrous copper sulphate and anhydrous sodium sulphate, but the best results were obtained with the last-mentioned material.

A chromatographic column, 20 × 1 cm, was packed with 4 g of anhydrous sodium sulphate (Merck, Darmstadt), forming a column 5 cm high. The sample to be

* Inst. Tisiol. Pneumol., Universidade do Brasil, P.O. Box 4485-ZC-21, Rio de Janeiro, Brazil.

examined was then added dropwise on to the top of the column, by means of a capillary pipette; air pressure was used to force the sample into the column. Sample volumes up to 1 ml can be treated in this way.

The water in the sample is absorbed by the anhydrous sodium sulphate and the proteins, salts and metabolic intermediates, including INH and its metabolic derivatives, are precipitated inside the column.

The INH and its derivatives can then be eluted from the column with a mixture of chloroform–diethylamine (90:10) (flow-rate = 2 ml/min). The eluate was evaporated to dryness on the water-bath, and the residue was taken up in a few microlitres of diethylamine.

Paper chromatography

Paper chromatography was carried out by the ascending technique, using Macherey-Nagel No. 261 filter paper. Control chromatograms were run with *n*-butanol saturated with 1 % ammonium hydroxide[1].

New solvent mixtures containing varying amounts of diethylamine, water and an organic solvent were also studied. The following solvents were tested: *n*-butanol, *n*-amyl alcohol, isoamyl alcohol, *n*-propyl alcohol, isopropyl alcohol, benzyl alcohol, ethyl alcohol, acetone and chloroform.

Complete sets of R_F values were determined for the solvent mixtures yielding the best separations for a sample containing INH and the isonicotinyl hydrazones of pyruvic acid and of acetaldehyde.

The sensitivity of a few more reagents towards INH and its derivatives was determined as described previously[5] by means of spot tests on filter paper. The following reagents were tested:

I. *Sodium nitroprusside.* 5 g of the salt are dissolved in a 10 % solution (v/v) of acetaldehyde in water; before using an equal volume of 2 % (w/v) sodium carbonate is added; after spraying the sample is heated at 120° for 10 min.

II. *Wachsmuth reagent*[6]. 2 g of quinhydrone are dissolved in 95 ml of ethanol plus 5 ml of pyridine; after spraying the sample is heated at 100° for 2 min.

III. *Ninhydrin.* 0.2 % (w/v) solution in acetone[7]; after spraying the sample is heated at 120° for 15 min.

IV. *Percheron reagent*[8]. 0.5 g barbituric acid dissolved in 100 ml ethanol containing 2 ml of 85 % phosphoric acid; after spraying and heating at 120° for 5 min the sample is detected by observing under a U.V. lamp.

V. Isatin. 1 g of the reagent is dissolved in 100 ml isopropanol containing 1 ml pyridine and 1.5 g zinc acetate; the sample is dried in an oven at 110°C and examined under a U.V. lamp.

VI. *Pyridine–acetaldehyde*[9]. The sampled is sprayed with a mixture containing equal parts of the reagents, dried at 110° and observed under a U.V. lamp.

RESULTS AND DISCUSSION

The above technique for the isolation of INH-derivatives by column chromatography was tested with mixtures containing INH, acetyl-INH and the INH hydrazones of pyruvic acid and acetaldehyde, dissolved in blood serum (1 % w/v concentrations of each component).

Elution was checked by paper chromatography, and it was found that 50 ml of chloroform–diethylamine were sufficient to completely elute the INH-derivatives, as shown in Table I.

TABLE I

ELUTION RATE OF VARIOUS INH-DERIVATIVES PURIFIED BY MEANS OF COLUMN CHROMATOGRAPHY

Fraction (10 ml)	*INH-derivatives present*			
	INH	*acetyl-INH*	*INHzone acet.*	*INHzone pyr.*
I	+	+	—	—
II	+	+	+	+
III	+	+	+	+
IV	—	—	+	+
V	—	—	+	+
VI	—	—	—	—

Blood samples, collected from mice 30 min after the intraperitoneal injection of INH (5 μg/g), were purified as described, and the derivatives eluted from the column were separated by two-dimensional paper chromatography[4]. Free INH, acetyl-INH and the hydrazones of pyruvic acid and acetaldehyde were identified by this method, confirming earlier results[1], together with isonicotinamide and di-isonicotinyl hydrazide.

Among the new diethylamine solvent mixtures tested, the following gave the best results: (I) *n*-butanol–diethylamine–water (40:10:satd.); (II) isopropyl alcohol–diethylamine–water (60:20:10); (III) benzyl alcohol–diethylamine–water (40:10:satd.); (IV) chloroform–diethylamine–water (40:20:satd.); (V) *n*-amyl alcohol–diethylamine–water (40:10:satd.). The R_F values found for the INH-derivatives are shown in Table II.

TABLE II

R_F VALUES OF INH-DERIVATIVES IN SOLVENT MIXTURES CONTAINING DIETHYLAMINE

Compound	*Solvent mixture*				
	I	*II*	*III*	*IV*	*V*
INH	0.53	1	0.53	0.38	0.22
Acetyl-INH	0.47	1	0.47	0.02	0.13
Pyruvic acid INHzone	0.42–0.56	1	0.41–0.53	0.09	0.08–0.19
Acetaldehyde INHzone	0.55	1	0.61	0.03	0.20
Isonicotinamide	0.76	1	0.67	0.58	0.52
Isonicotinic acid	0.54	1	0.49	0.14	0.18
Di-INH	0.55	1	0.60	0.03	0.20

As can be seen, solvent II (isopropyl alcohol–diethylamine–water) is particularly useful for the direct chromatographic separation of INH derivatives from wet samples[4]; solvent IV (chloroform–diethylamine–water) is suitable for separating INH, isonicotinic acid and isonicotinamide from the remaining derivatives.

The results for the sensitivity of the spray reagents described under "materials

and methods" are shown in Table III, together with the colours given by the various derivatives.

Reagent I (sodium nitroprusside) was found to have good sensitivity to all derivatives, giving characteristic colours with INH, the pyruvic hydrazone and isonicotinic acid. Reagent IV (barbituric acid) was shown to be specific for the hydrazones,

TABLE III

SENSITIVITY OF VARIOUS REAGENTS TOWARDS INH AND ITS METABOLIC DERIVATIVES

INH-derivative	*Reagent*					
	I	*II*	*III*	*IV*	*V*	*VI*
			Sensitivity			
INH	2	2	2	—	4	30
Acetyl-INH	2	5	20	—	4	—
Pyruvic acid INHzone	2	2	2	0.5	8	20
Acetaldehyde INHzone	2	2	2	20	4	10
Isonicotinamide	2	10	20	—	—	—
Isonicotinic acid	2	2	1	—	—	—
Di-INH	2	2	2	—	4	50
			Colour			
INH	Orange	Brownish	Yellow	—	Brown	Yellow
Acetyl-INH	Brownish	Brownish	Yellow	—	Yellow	—
Pyruvic acid INHzone	Bordeaux	Bordeaux	Brown	Brown	Brown	Blue
Acetaldehyde INHzone	Brownish	Brownish	Orange	Brown	Yellow	Yellow
Isonicotinamide	Brownish	Bordeaux	Yellow	—	—	—
Isonicotinic acid	Yellow	Bordeaux	Yellow	—	—	—
Di-INH	Brownish	Yellow	Orange	—	—	—

having a high sensitivity to the pyruvic derivatives. Reagent VI (pyridine–acetaldehyde) although being specific for INH, di-INH and the hydrazones, had lower sensitivity. Reagents II (quinhydrone) and III (ninhydrin) showed good sensitivity towards most IHN-derivatives, except acetyl-INH and isonicotinamide. Reagent V (isatin) was found to be specific for the derivatives containing the hydrazine moiety; sensitivity, however, was not very high.

ACKNOWLEDGEMENTS

The present work was carried out at the Department of Biochemistry of the Institute of Phthisiology and Pneumology of the University of Brazil, in collaboration with the Central Laboratory of Tuberculosis of the State of Guanabara and with the support of the National Research Council of Brazil.

SUMMARY

The authors studied the isolation of INH-derivatives from samples of biological material, such as plasma and serum, by means of chromatography on columns of anhydrous sodium sulphate, followed by elution with chloroform–diethylamine

(90:10). Fairly large volumes of material can be treated, the eluted derivatives being easily concentrated by evaporating to dryness.

Solvent mixtures for paper chromatography containing diethylamine were also studied. The solvent mixture chloroform–diethylamine–water (40:20:satd.) was found to give a good separation of INH, isonicotinic acid and isonicotinamide from the remaining derivatives.

Among the new reagents tested for INH-derivatives, good results were obtained with sodium nitroprusside, quinhydrone and ninhydrin. Specific reactions were obtained with barbituric acid (hydrazones), pyridine–acetaldehyde (INH, di-INH and the hydrazones) and isatin (derivatives containing the hydrazine moiety).

REFERENCES

1 R. C. R. BARRETO, *J. Chromatog.*, 7 (1962) 82.
2 R. C. R. BARRETO AND D. B. MANO, *Biochem. Pharmacol.*, 8 (1961) 403.
3 R. C. R. BARRETO AND S. O. S. SABINO, *J. Chromatog.*, 9 (1962) 180.
4 R. C. R. BARRETO AND S. O. S. SABINO, *J. Chromatog.*, 11 (1963) 344.
5 H. S. R. BARRETO AND R. C. R. BARRETO, *J. Chromatog.*, 4 (1960) 153.
6 H. WACHSMUTH, *J. Pharm., Belgique*, 11 (1956) 86.
7 G. TOENNIES AND J. J. KOLB, *Anal. Chem.*, 23 (1951) 283.
8 F. PERCHERON, *Bull. Soc. Chim. Biol.*, 44 (1962) 1162.
9 O. FURTH AND H. HERRMANN, *Biochem. Z.*, 280 (1935) 448.