ISOLATION AND IDENTIFICATION OF THE METABOLIC PRODUCTS OF ISONICOTINIC ACID HYDRAZIDE FROM BLOOD SAMPLES BY MEANS OF PAPER CHROMATOGRAPHY

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INTRODUCTION

The identification of the metabolic products of isonicotinic acid hydrazide (INH) in blood samples is specially important for the investigation of the mode of action of that drug. It presents some difficulties, however, as the usual deproteinization procedures must be avoided owing to the instability of some of the metabolites. The same is true of the concentration and drying procedures, when all care must be taken to minimize the loss of material.

This led us to apply descending paper chromatography for the isolation of some of the more important metabolic derivatives of INH, previously separated from protein material by means of centrifugally accelerated ultrafiltration, and to use two-dimensional paper chromatography for their identification.

MATERIALS AND METHODS

Centrifugal ultrafiltration

Collodion jackets were prepared by pouring a 4% solution into 50 ml centrifuge tubes, draining them until dry and filling them with water. After 1 hour the jackets could be easily removed and were ready for use. Blood samples (5 ml) from INH-treated animals, containing an anti-clotting agent (sodium oxalate, in most cases), were placed in the collodion jackets, which were tied and suspended inside 50 ml centrifuge tubes by means of gauze bags (held halfway up the tubes by means of rubber bands clasping the folded gauze around the necks of the tubes). In the case of smaller samples the volume was adjusted to 5 ml with isotonic NaCl solution. Centrifugation at 1,500 r.p.m. during 1 hour yielded about 3 ml of protein-free filtrate from each sample.

Chromatographic purification

The protein-free filtrates were evaporated to dryness in the water-bath (80°) under vacuum, and the dry residues were dissolved in 0.1 ml of 1% (w/v) sodium carbonate.

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This facilitates the solubilization of the isonicotinyl-hydrazones and the separation of the INH-metabolites from contaminating material.

The purification of the filtrates was accomplished by means of descending paper chromatography of the alkaline solution (applied *in totum* as a streak 5 × 0.5 cm), using Macherey-Nagel No. 261 filter paper and ISHERWOOD's[1] solvent: propanol–conc. ammonium hydroxide (70:30). This separates the sodium salts[2] and most of the contaminants in the first halves of the chromatograms, and INH and its derivatives (with the exception of sodium isonicotinate and pyridoxal phosphate isonicotinyl-hydrazone) in the second.

The chromatograms were dried in a current of air, at room temperature, and observed under the U.V. lamp. The regions containing INH and metabolites were cut out and eluted overnight with distilled water. The eluates were evaporated to dryness (80°, under vacuum) and the residues were dissolved in a few microliters of distilled water.

Chromatographic identification of the metabolites

The identification of INH and its metabolic products was carried out by means of two-dimensional paper chromatography of the purified samples. Table I shows the

TABLE I

R_F VALUES OF SOME DERIVATIVES OF ISONICOTINIC ACID HYDRAZIDE IN VARIOUS SOLVENTS

Compound	R_F *values*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
INH	0.85	0.61	0.52	0.83	0.78	0.61	0.90	0.71
Acetyl-INH	0.87	0.57	0.35	0.92	0.43	0.61	0.81	0.71
Acetaldehyde isonicotinyl-hydrazone	0.88	0.38	0.24	0.89	0.42	0.68	0.82	0.71
Na pyruvate isonicotinyl-hydrazone	0.28	0.40	0.20	0.58	tail	0.32	0.70	0.49
Pyridoxal phosphate isonicotinyl-hydrazone	0.06	0.00	0.19	0.03	0.02	0.08	0.04	0.03

Solvent	*Composition*	*Ref.*
1	Water-saturated *n*-butanol	3
2	Isopropanol–1% ammonium hydroxide (20:3)	3
3	*n*-Butanol satd. with 1% ammonium hydroxide	3
4	Isopropanol–water (85:15)	4
5	Isoamyl alcohol satd. with 0.5 *N* acetic acid	4
6	*n*-Butanol satd. with 0.5 *N* acetic acid	4
7	Propanol–ammonium hydroxide (70:30)	1
8	*n*-Butanol–ethanol–water (40:10:50)	5

behaviour of INH and some of its metabolites on paper chromatograms run by the ascending technique on Macherey-Nagel No. 261 filter paper.

From the table it can be seen that solvents 2 (isopropanol–1% ammonium

hydroxide, 20:3) and 3 (*n*-butanol saturated with 1% ammonium hydroxide) are the most suitable. The position of the spots was ascertained as follows: (a) observation of the dry chromatograms under the U.V. lamp (pyruvic acid isonicotinyl-hydrazone and acetyl-INH—absorption spots; acetaldehyde isonicotinyl-hydrazone—orange-

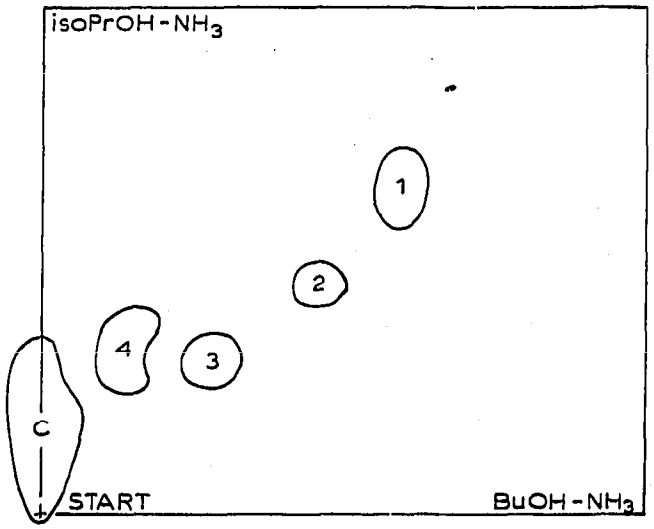

Fig. 1. Two-dimensional paper chromatogram of INH and some of its metabolites: (1) INH; (2) acetyl-INH; (3) acetaldehyde isonicotinyl-hydrazone; (4) pyruvic acid isonicotinyl-hydrazone; (c) contamination.

yellow spots); (b) paper chromatograms kept in an atmosphere of cyanogen bromide plus ammonia during 1 hour (INH, acetyl-INH and acetaldehyde isonicotinyl-hydrazone yield yellow spots).

RESULTS AND DISCUSSION

Fig. 1 shows the results obtained after the application of the described technique to the separation of INH and some of its derivatives, previously added to a sample of rabbit blood (blood sample, 5 ml; added metabolites, 50 μg each).

The application of the same technique to samples of blood collected from mice and from rabbits 1 hour after the injection of 100 mg of INH per kg body weight showed the presence of six metabolites (two unidentified), besides five spots of contaminating material.

The presence of the isonicotinyl-hydrazone of acetaldehyde in blood samples was confirmed by further experiments, using ^{14}C labelling of the metabolic derivatives, and will be discussed elsewhere.

It must be kept in mind that the chromatographic isolation of the derivatives of INH as described leads to the loss of the derivatives with low R_F values in propanol–ammonia, such as pyridoxal phosphate isonicotinyl-hydrazone (see Table I) and isonicotinic acid (which has an R_F value around 0.13).

The techniques reported here present the advantages of the absence of deleterious effects upon the metabolic derivatives and the possibility of separating a large number of compounds that are otherwise indistinguishable.

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SUMMARY

The method here described was devised for the study of the metabolic products of isonicotinic acid hydrazide from blood samples.

By means of centrifugal ultrafiltration and descending paper chromatography the authors were able to isolate and purify some of the more unstable derivatives of INH, which were identified by means of two-dimensional paper chromatography.

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