CHROMATOGRAPHIC STUDIES ON ISONICOTINIC ACID HYDRAZIDE AND ITS METABOLIC DERIVATIVES

III. NEW SOLVENTS AND NEW TECHNIQUES

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INTRODUCTION

On previous occasions[1,2] we have studied the paper chromatography and paper electrophoresis of isonicotinic acid hydrazide (INH) and its metabolic derivatives, isolated from blood samples by means of centrifugal ultrafiltration[3,4]. Though efficient, this technique of purification has drawbacks, such as the occasional rupturing of the dialysis bags and the need of heat to concentrate the filtered material.

The recent paper by DAVIS, DUBBS AND ADAMS[5], on an elution–concentration method, led to the development of a technique for the chromatographic isolation of INH-derivatives from wet samples of blood serum. Furthermore, new solvents have been used for the separation of these compounds and papers impregnated with ethylenediaminetetraacetic acid (EDTA) have been tried with good results.

MATERIALS AND METHODS

Chromatographic isolation of INH and derivatives

Blood was drawn from the INH-treated animal and serum was separated as usual, only a few microliters being needed[6]. The serum was then streaked along a line parallel to and at 10 cm from the top of a strip of Macherey-Nagel No. 261 filter paper measuring 40 × 3 cm. No more than 10 μl of serum should be applied to the paper, and the streak must run from edge to edge of the strip, in order to avoid constriction of the spots during the chromatographic run and to force the solvent through the sample. Greater amounts of serum tend to block the passage of the solvent, owing to the precipitation of the proteins by the organic phase.

Paper chromatography was then carried out by the descending method, through the wet sample of serum (drying of the sample prevents the displacement of the solvent). Attention must be paid to the fact that after the paper strip is placed in the chromatographic chamber the initial distance between the solvent and the sample must be about 2 cm, in order to avoid massive precipitation of proteins at the beginning of the run, which would be caused by a solvent front too rich in organic phase.

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Any one of the solvents already studied for the paper chromatography of INH-derivatives[1,2] can be used in the present case. Some of the best results, however, were obtained with a mixture of pyridine and water (65:35), especially when the procedure described above was combined with the elution–concentration method of DAVIS, DUBBS AND ADAMS[5].

In this last case, instead of pressing the filter paper between glass plates, as described by those authors, the following procedure was adopted: a strip of Macherey-Nagel No. 261 filter paper 6 cm wide was cut in the necessary number of segments and the samples were applied to the paper (one on each segment), which was then covered with a bent sheet of aluminium foil, as shown in Fig. 1.

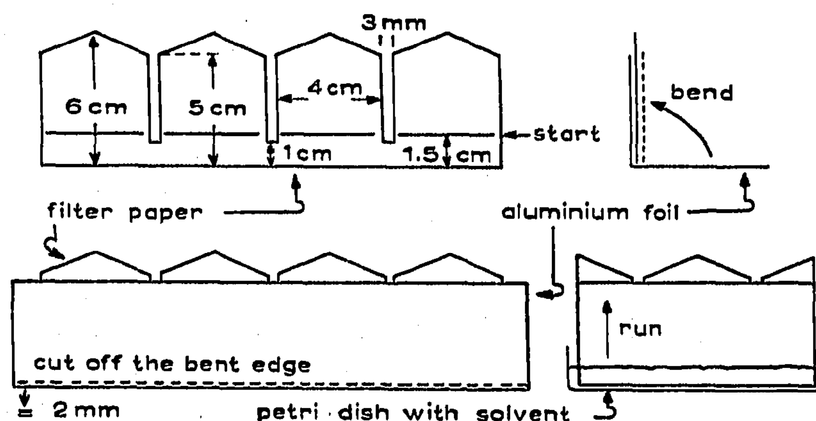

Fig. 1. Setup for the direct chromatographic isolation of INH-derivatives from wet samples of blood serum.

This setup was then run as an ascending chromatogram, after clipping it into a cylinder and cutting off the bent edge of the foil. INH and its derivatives are concentrated at the top of each segment by the evaporation of the solvent, and are easily eluted with a few microliters of pyridine.

Paper chromatography

As before, Macherey-Nagel No. 261 filter paper was used in all the experiments described. In the case of EDTA-impregnated papers the sheets of filter paper were soaked in a 0.2 M solution of ethylenediaminetetraacetic acid (the excess of which was blotted out with filter paper) and dried afterwards in the oven at 120°. In the case of impregnated papers the solvents were saturated with 0.2 M EDTA.

The spots were localized as before[2], using the sequential procedure U.V.–BrCN/NH_3–GREULACH-HAESLOOP reagent[7].

RESULTS AND DISCUSSION

The method described for the chromatographic isolation of INH-derivatives from wet samples of blood serum was found to be useful in all cases where the amount of the drug administered to the animal was large enough to be detected in the final paper chromatogram, since the volume of the serum sample applied to the paper had to be limited to 10 µl.

Among the solvents commonly used in paper chromatography, pyridine was found to be the only one capable of dissolving all the INH-derivatives with which we were concerned. This led us to study some of the pyridine-containing solvent mixtures already in use, as shown in Table I.

TABLE I

R_F VALUES FOR SOME INH-DERIVATIVES IN SOLVENT MIXTURES CONTAINING PYRIDINE

Compound	*R_F values*			
	1	*2*	*3*	*4*
INH	0.60	0.89	0.74	0.73
Acetyl-INH	0.66	0.92	0.71	0.54
Acetaldehyde INHzone	0.69	0.92	0.76	0.58
NH_4 pyruvate INHzone	0.42/0.59	0.89	0.44/0.59	0.68
NH_4 isonicotinate	0.37	0.87	0.42	0.55
Isonicotinamide	0.67	0.88	0.69	0.72
Diisonicotinyl hydrazide	0.71	0.92	0.74	0.55

Solvent	*Composition*	*Ref.*
1	Pyridine–amyl alcohol–water (40:35:30)	8
2	Pyridine–water (65:35)	9
3	Isoamyl alcohol–pyridine–water (20:25:20)	10
4	Pyridine–isoamyl alcohol–1.6 N NH_4OH (20:14:20)	11

reagent[7] omits the spots of isonicotinic acid and isonicotinamide, making it easier to identify the remaining derivatives.

One of the main problems encountered in the paper chromatography of INH is the frequent formation of "tails", which we tried to avoid by impregnating the paper with a chelating agent, such as EDTA. The results obtained with some EDTA-saturated solvents are shown in Table II. As can be seen, the R_F values found for the INH spots were lower than those obtained with the corresponding water-saturated mixtures[1,2], and higher for the other derivatives. INH-tailing, on the other hand, was either very much reduced or completely eliminated.

TABLE II

R_F VALUES FOR SOME INH-DERIVATIVES IN EDTA-IMPREGNATED FILTER PAPER

Compound	*R_F values*				
	1	*2*	*3*	*4*	*5*
INH	0.66	0.79	0.84	0.76	0.71
Acetyl-INH	0.79	0.87	0.81	0.77	0.58
Acetaldehyde INHzone	0.83	0.84	0.85	0.82	0.58
NH_4 pyruvate INHzone	0.09	0.06	0.56	0.57	0.05
	0.42	0.37	0.76	—	—
NH_4 isonicotinate	0.48	0.34	0.57	0.50	0.23
Isonicotinamide	0.71	0.83	0.80	0.76	0.62
Diisonicotinyl hydrazide	0.80	0.85	0.82	0.85	0.58

Solvent	*Composition*
1	Pyridine–amyl alcohol–0.2 *M* EDTA (40:35:satd.)
2	*n*-Butanol satd. with 0.2 *M* EDTA
3	*n*-Butanol–ethanol–0.2 *M* EDTA (40:10:satd.)
4	Isoamyl alcohol–pyridine–0.2 *M* EDTA (20:25:satd.)
5	Isoamyl alcohol satd. with 0.2 *M* EDTA

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SUMMARY

The authors devised a method for the direct chromatographic isolation of derivatives of isonicotinic acid hydrazide from wet samples of blood serum. This method is based on forcing the solvent through the sample, applied as a streak, and on the concentration of the isolated material at the top of the filter paper, caused by the evaporation of the solvent.

In addition pyridine-containing solvents were studied and papers impregnated with ethylenediaminetetraacetic acid were tested, in order to obtain better separations of the derivatives and to avoid tailing of the isonicotinic acid hydrazide spots.

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