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Some technical improvements in the paper chromatography of keto acid 2,4-dinitrophenylhydrazones

Alpha keto acids are commonly studied in biological samples, as their 2,4-dinitrophenylhydrazones[1–6] (DNP-hydrazones). In the usual extraction procedures, DNP-hydrazones of keto acids are extracted into an organic solvent followed by re-extraction into an alkaline aqueous layer. After acidification of this aqueous layer they are again taken up into an organic solvent. The last step is accompanied by loss of some of the DNP-hydrazones[3] and it has been dispensed with in certain modifications[1,3]. In these modified methods, however, one has to spot 300–500 μl of an aqueous solution, which is rather tiresome. In the method to be described in this paper DNP-hydrazones of keto acids are taken up from sodium carbonate solution without acidification into small amounts of a triethylamine–pyridine mixture (2:1). The final volume obtained in this way is comparable to the one obtained in the modifications referred to above, but it is much easier to apply.

A solvent is also described which separates the DNP-hydrazones of common keto acids in about two hours, as compact spots, almost irrespective of the area over which the sample is spotted.

Experimental

DNP-hydrazones of keto acids are prepared according to MCARDLE[1].

The DNP-hydrazones are first taken up into a mixture of chloroform–ethyl alcohol (4:1) and subsequently into 1 *N* sodium carbonate solution[2]. Two extractions with 3 ml and 2 ml sodium carbonate solution, respectively, are made to obtain the DNP-hydrazones in 5 ml of the solution, corresponding to 2 ml urine or 5 ml blood.

The sodium carbonate solution, taken in a 10.5 × 1.4 cm stoppered tube, is cooled down to 0–4° and sufficient sodium carbonate is added, to obtain a saturated solution at room temperature. At 35° about 1.5 g may be needed for the purpose.

After the tube attains the room temperature DNP-hydrazones are extracted into a triethylamine–pyridine mixture (2:1). First two extractions are made, each with 0.5 ml of the mixture and subsequently two or more, each with 0.25 ml. For every extraction vigorous shaking is necessary. The layers separate out without any difficulty. In the final extraction the upper layer should be colourless.

100–500 μl of the triethylamine–pyridine extract is spotted for chromatography.

TABLE I

RECOVERIES OF DNP-HYDRAZONES OF KETO ACIDS FROM THE SODIUM CARBONATE SOLUTION WITHOUT ACIDIFICATION AND AFTER ACIDIFICATION

Solvent: triethylamine–ether–water–pyridine (15:10:5:5) at 32°.
A = Amount extracted into triethylamine–pyridine mixture (2:1) without aging the sodium carbonate extract, extract spotted immediately; B = amount extracted into ethyl acetate after acidification[2] and under conditions as in A; C = amount extracted into triethylamine–pyridine mixture under conditions as in A but the extract spotted after having been kept at 35° for 45 min; D = amount extracted into triethylamine–pyridine mixture (2:1) after keeping the sodium carbonate extract for 45 min at 35°, extract spotted immediately; E = amount extracted into ethyl acetate after the acidified extract was kept for 45 min at 35°, extract spotted immediately.

Compound (DNP-hydrazone of)	R_F	*Amounts extracted (%) from the sodium carbonate solution under different conditions**				
		A	*B*	*C*	*D*	*E*
α-Ketoglutaric acid	0.25	100	95.6	100	100	33.3
Oxaloacetic acid	0.25	100	85.7	100	100	62.0
Phenylpyruvic acid-1	0.51	100	93.0	100	100	69.8
Phenylpyruvic acid-2	0.78	100	91.7	100	100	58.3
Pyruvic acid-1	0.41	100	86.4	100	100	63.7
Pyruvic acid-2	0.62	100	90.0	100	100	65.0

* Recovery under A is arbitrarily designated as 100% and recoveries under other heads are expressed as percent of recovery under A.

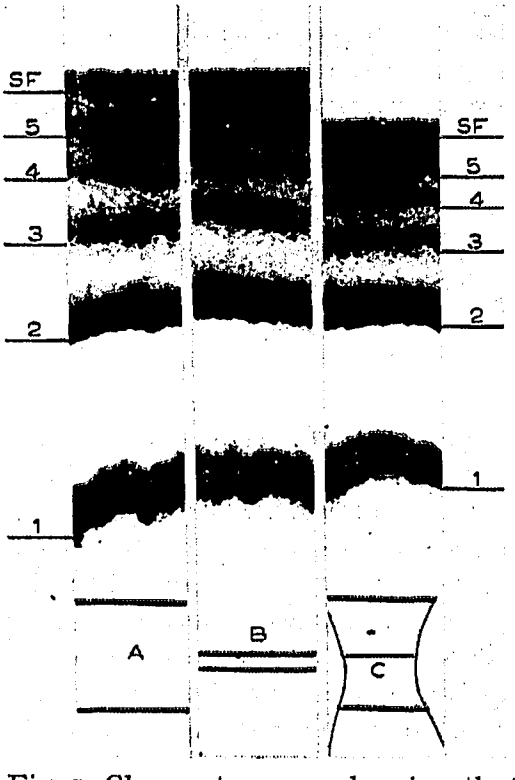

Fig. 1. Chromatograms showing that the spot size has no effect on the compactness of the bands obtained with the solvent triethylamine–ether–pyridine–water (15:10:5:5). Thick lines near the origin enclose the areas over which similar amounts of an extract have been applied in the strips A, B and C. In C a special strip has been used[3]. Papers have been given a descending run of 1.5 h at 32°. Bands are as follows: 1 = α-ketoglutaric acid; 2 = pyruvic acid; 3 = phenylpyruvic acid; 4 = pyruvic acid; 5 = phenylpyruvic acid; SF = solvent front.

Results and discussion

There is a definite loss of DNP-hydrazones of keto acids, in the final step of procedures in which extraction into an organic solvent is made, after making the aqueous phase acidic. The losses are related to the time DNP-hydrazones are kept in the acidic aqueous layer before extraction into the organic solvent. These losses are prevented in the method described (see Table I) and also in some previously described extraction procedures[1,3]. In the present method, however, there is an added advantage that the DNP-hydrazones are taken up in a volatile solvent for spotting.

Stability of DNP-hydrazones in the triethylamine–pyridine mixture is similar to that in sodium carbonate solution (see Table I).

The solvent mixture triethylamine–ether (peroxide free)–water–pyridine (15:10:5:5, v/v) separates the DNP-hydrazones of α-ketoglutaric acid, pyruvic acid and phenylpyruvic acid in less than two hours. The DNP-hydrazone of oxaloacetic acid, however, overlaps that of ketoglutaric acid. In preparing this solvent first the three constituents are mixed and pyridine is then added gradually while shaking to obtain a clear solution.

The spots obtained by this solvent are compact and their size is almost unrelated to the size of the spot applied (see Fig. 1). The best results with the solvent are obtained when the room temperature lies between 20–30°.

In this solvent some of the DNP-hydrazones have different colours on the wet chromatograms. The DNP hydrazone of one of the spots of phenylpyruvic acid (R_F 0.51) has a brownish colour which changes to a yellowish one when the chromatogram dries. Similarly in urine samples from patients with infantile cirrhosis a purple spot (R_F 0.6) is seen which becomes orange-brown as the paper dries. The spot remains to be identified.

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