Journal of Chromatography, 105 (1975) 215–218

CHROM. 8025

Note

Paper chromatography of acetamide and its application to metabolic studies

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(Received September 13th, 1974)

The chromatographic purification and selective detection of acetamide is important since it can be both produced and metabolised by certain micro-organisms[1–3]. The amide could be partially separated from other cell components by either gas–liquid chromatography[4–8] or thin-layer chromatography[9] but these techniques are inconvenient for radioactive tracer studies. Fortunately, the compound, despite its volatility (b.p. 222°) can be chromatographed on paper[10–12], so we investigated this possibility more seriously.

EXPERIMENTAL

Samples were spotted onto Whatman No. 3MM paper at 45° and chromatographed at 20–25° until the amide had migrated approximately 30 cm. The papers were normally dried for 2 h at 50° but phenol–water chromatograms were heated for 24 h at this temperature.

Acetamide can be detected, after chromatography, using either the phenol–hypochlorite reaction[10] or Ehrlich's reagent[13], the latter giving a yellow colouration with as little as 20 $\mu g/cm^2$. However, we preferred the more sensitive and specific iron(III) hydroxamate test, widely used for revealing acid amides[9,11,12,14]. A modified form of this reaction was able to detect 1.5 μg acetamide per cm^2.

Thus, spots were located by dipping the chromatograms twice through saturated hydroxylammonium chloride in methanol and drying them for 7 min at 50° after each application. The papers were then heated for 20 min at 80° and dipped through 2% (w/v) anhydrous iron(III) chloride in acetone. Amides appeared as purple spots on a yellow background as the acetone evaporated. Hydroxylammonium chloride in methanol is a health hazard. Moreover, the reagent should not be allowed to run along the paper since it can dissolve and therefore displace the acetamide zones.

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The amide ran as a sharp, discreet spot in all the solvents investigated and its chromatographic properties are given in Table I. The total loss incurred during chromatography is probably less than 20% if the papers are dried for 2 h at 50°. The more intensive drying needed to eliminate phenol-based solvents, 24 h at 50°, increases the loss to approximately 50% (Table II). Recoveries after two-dimensional chromatography, as specified in Fig. 1, appear to be as low as 40%. However, this may be partly due to suppression of the hydroxamate reaction by solvent residues. Certainly, when the test is applied to chromatograms run in *n*-butanol–acetic acid–water its sensitivity doubles if the papers are pre-dried for 24 h rather than 2 h at 50°.

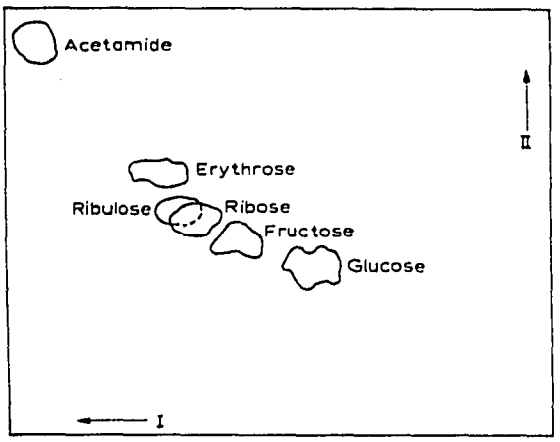

Fig. 1. Chromatographic map showing the position of acetamide relative to that of selected non-ionic metabolites. This map was taken from an actual chromatogram on which acetamide was revealed by local treatment with the hydroxamate reagent and the sugars with an alkaline silver reagent[16]. Calvin and Bassham[17] have published more comprehensive maps for this solvent system. First dimension: solvent, phenol–water (100:39, w/v); time of run, 15 h; dried for 8 h at 50°. Second dimension: solvent, *n*-butanol–propionic acid–water (92:47:61, v/v); time of run, 16 h; dried for 2 h at 50°.

If labelled acetamide is to be detected in cellular extracts, it is essential to add 100–1000 μg of "cold" carrier. Labelled anionic and cationic substances may then be eliminated by ion exchange as detailed in Table II. These studies confirmed that acetamide interacts weakly with cation-exchange resins[18] but showed that it could be recovered satisfactorily by thorough washing. They also illustrated the difficulty of recovering the amide quantitatively from aqueous solution. Evaporation in a vacuum desiccator containing concentrated sulphuric acid and solid sodium hydroxide must definitely be avoided as the losses can exceed 95% at the 1000-μg level; presumably acetamide is dehydrated to the very volatile methyl cyanide under these conditions. However, small volumes of acetamide in methanol may be evaporated to dryness under a stream of nitrogen with little loss.

Acetamide can be easily separated from all common non-ionic metabolites by two-dimensional chromatography (Fig. 1); indeed one-dimensional chromatography will be sufficiently specific for many applications.

Labelled acetamide has been successfully located by conventional radioautography; 500-μg samples run on two-dimensional chromatograms have been

TABLE I

CHROMATOGRAPHIC DATA OBTAINED BY PAPER CHROMATOGRAPHY OF ACETAMIDE

Solvent	R_F*	$R_{Glucose}$*	*Minimum detectable quantity after chromatography (μg)***
Organic phase of *n*-butanol–90% formic acid–water (100:24:100, v/v)	0.61, 0.58	8.0, 6.7	5
n-Butanol–acetic acid–water (90:10:29, v/v)	0.63, 0.61, 0.61, 0.62, 0.65	4.4, 3.9	8
n-Butanol–propionic acid–water (92:47:61, v/v)	0.73, 0.72	3.0, 2.8	3
Organic phase of *tert.*-amyl alcohol–90% formic acid–water (80:10:40, v/v)	0.64, 0.66	5.1, 4.4	3
Phenol–water (100:39, w/v)	0.95, 0.97	2.2, 2.2	8

* The values quoted were always obtained from separate chromatograms, normally run in different laboratories at different times.

** The minimum detectable quantity after two-dimensional chromatography (Fig. 1) was 20 μg when the amide had migrated a total of 60 cm.

TABLE II

LOSSES OF ACETAMIDE INCURRED DURING CHROMATOGRAPHY AND RELATED OPERATIONS*

Procedure	*Initial weight of amide taken (μg)*	*Recovery (%)*
Paper chromatography		
Samples spotted directly onto 1.5-cm² areas of 3MM paper were heated:		
(a) at 100° for 4 h	5	65 ± 15
(b) at 50° for 27 h	5	55 ± 15
Samples were chromatographed in *n*-butanol–propionic acid–water as described in Table I	3	90 ± 20
Ion exchange fractionation		
Aqueous acetamide (1 ml) was passed through a 5-cm × 1-cm-diameter column of Zeo-Karb 225 (4.5% divinylbenzene, 50–100 mesh) in the H^+ form. This strong cation-exchange resin was then washed with 100 ml water.	1000	90 ± 3
Aqueous acetamide (1 ml) was passed through a 5-cm × 1-cm-diameter column of Dowex 1 (20–50 mesh) in the CH_3COO^- form. This strong anion-exchange resin was then washed with 100 ml water.	1000	100 ± 3
Aqueous acetamide (100 ml) was evaporated to dryness in a rotary film evaporator at 50°.	500	53 ± 4
	1000	57 ± 1
	2000	60 ± 1
Methanolic solution of acetamide (5 ml) was evaporated to dryness at 20° in a stream of nitrogen.	1000	90 ± 5
Complete fractionation procedure: Aqueous acetamide (1 ml) was allowed to flow through both colums in series and the combination was then washed with 100 ml water. The effluent was evaporated to dryness at 50°, as described, the residue being taken up in methanol and re-evaporated.	1000	38 ± 4

* Acetamide on paper was estimated by visual comparison with freshly spotted standards, using the test given in the text. Where appropriate, spot areas were taken into account. Amide in solution was determined by the method of Soloway and Lipschitz[15], optimised for the measurement of acetamide. Thus the aqueous sample (1 ml) was heated with 1 *M* hydroxylammonium chloride in glycerol (2 ml) for 60 min at 110°. After cooling, the product was mixed with 2.5% (w/v) anhydrous iron(III) chloride in 2 *N* HCl (0.5 ml) and its optical density was measured at 520 nm.

exposed to X-ray film for up to six weeks and then successfully detected with the hydroxamate reagent. Acetamide spots, located by comparison with standards, have also been satisfactorily eluted from 3MM paper by vigorous agitation (vortex mixer) with 5 ml 70% v/v ethanol for 2 min and assayed for radioactivity by scintillation counting.

These methods not only enable [^{14}C]acetamide to be detected in a highly specific way but are of value for quantitative comparative investigations.

ACKNOWLEDGEMENT

Thanks are due to Miss G. Hepburn, who optimised the method of Soloway and Lipschitz for the measurement of acetamide.

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