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Anomalous behaviour of radioactive folic acid on thin-layer chromatography

The use of thin-layer chromatography to determine the purity and identity of trace amounts of organic compounds is well established. We now report an example in which the chromatographic behaviour changed with concentration of the substance under investigation.

Folic acid (pteroyl-L-monoglutamic acid) may be readily detected by TLC on cellulose powder in amounts greater than 5 μg by its appearance as a dark absorbent spot when viewed in 254 mμ light. With butanol–acetic acid–water (4:1:5, upper phase pH 2.6) as developing solvent, folic acid has an R_F value of 0.0 and this conveniently distinguishes it from its fluorescent decomposition products which move away from the origin.

When [2-^{14}C]folic acid (0.45 μg/μl; specific activity 50.3 mCi/mmole; Radiochemical Centre, Amersham) was assayed for purity in this system a major spot, as determined by autoradiography, of an R_F value of 0.42 was obtained with nothing at the origin. This led to the initial conclusion that the sample of folic acid had extensively decomposed although this radioactive material was not identical with the anticipated decomposition products. When TLC of the radioactive compound (0.45 μg/μl) was carried out in 0.1 *M* phosphate buffer (pH 7.0) and in propanol–1% aq. ammonia (2:1) the major radioactive spot had the same chromatographic behaviour as cold folic acid (5 μg/μl). When folic acid was added to the radioactive folic acid so as to produce a wide range of concentrations and TLC was carried out in butanol–acetic acid–water (4:1:5, upper phase) the R_F values of the major radioactive species varied as shown in Tables I and II.

TABLE I

R_F VALUES OF MIXTURES OF RADIOACTIVE AND NONRADIOACTIVE FOLIC ACID
[2-^{14}C]folic acid 0.45 μg/μl.

Nonradioactive folic acid added	R_F
0.0	0.42
8 μg/2 μl	0.00
20 μg/3 μl	0.00
40 μg/5 μl	0.00

TABLE II

R_F VALUES OF MIXTURES OF RADIOACTIVE AND NONRADIOACTIVE FOLIC ACID
Mixture of 0.5 μg [2-^{14}C]folic acid and 10–15 μg nonradioactive folic acid.

Volume of solution applied (μl)	R_F
2	0.0
10	0.0
50	0.50

These data show that folic acid in butanol–acetic acid–water has an R_F value of 0.0 when applied in concentrated solutions (more than 1 $\mu g/\mu l$) and an R_F value of 0.42 when applied in dilute solution (0.5 $\mu g/\mu l$).

As these differences in chromatographic behaviour could be attributed to chelation of the folic acid with metals, chromatograms were run in butanol–acetic acid–water saturated with ethylene diamine tetracetic acid and butanol–acetic acid–water containing a crystal of sodium sulphide[1]. Again dilute solutions of folic acid had an R_F value of 0.49 and concentrated solutions an R_F value of 0.00.

With 3% aqueous acetic acid (adjusted to pH 3.4 with NaOH) dilute solutions (0.2–0.3 $\mu g/\mu l$) of folic acid gave an R_F value of 0.20–0.30 and concentrated solutions (5–7.5 $\mu g/\mu l$) an R_F value of 0.00; the behaviour was not altered by the addition of ethylene diaminetetracetic acid.

With 3% aqueous ammonium chloride at pH 4.0 dilute solutions of folic acid (0.45 $\mu g/\mu l$) gave two spots (R_F 0.04 and R_F 0.27) coalescing into one R_F (0.04) at higher concentrations (7.5 $\mu g/\mu l$) but at pH 5.5 only one spot (R_F 0.30–0.40) was obtained at all concentrations.

The absence of any effect of adding sodium sulphide or ethylene diamine-tetra-acetic acid establishes that this anomalous effect is not due to chelation of the folic acid. As the variation in R_F values with concentration is found only in acidic solvent systems and disappears when the pH of the 3% aqueous ammonium chloride system is changed from 4.0 to 5.5 this behaviour is due to the non-ionised acid. The variation in chromatographic behaviour with concentration of folic acid in the acidic solvents used is caused by the association of the acid, the species present at low concentrations and having the higher R_F value being a monomer and the species present at higher concentrations and having the lower R_F value being the associated form. The association of the folic acid molecules could be caused by intermolecular hydrogen bonding between the non-ionised carboxyl groups but this seems unlikely as the hydrogen bonds of non-ionised carboxyl groups in aqueous solutions are made preferentially with the solvent molecules and not with each other[2]. There is strong intermolecular interaction between the pteridine rings of folic acid as evidenced by its infusibility and insolubility in all solvents but aqueous alkaline solutions[1]. This intermolecular interaction would be a reasonable explanation for the association of folic acid in higher concentrations in acidic solvent systems. A similar association in aqueous solutions has been established for purines where it has been shown that purines associate with the molecular planes parallel to each other held by interactions between the π-electrons of each ring[3,4].

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