Double spot formation in chromatography of imidazolepropionic acid

Recently more attention has been given in biology and medicine to imidazolepropionic acid. BALDRIDGE AND TOURTELLOTTE[1] found the substance in rat urine after a histidine load was given. AUERBACH *et al.*[2] identified imidazolepropionic acid in the urine of an infant with histidinemia. SEN *et al.*[3] showed that imidazolepropionic acid is a normal constituent of human urine, and in the rat is derived from urocanic acid. Its role in bacterial metabolism has been investigated[4,5].

In our laboratory one of the solvents previously used for this compound[1,3] has consistently produced double zones on paper chromatograms. Since it is usually assumed that pure organic compounds give single spots on paper chromatograms, we first questioned the purity of the compound and then explored the possibility of of multiple spot formation. This report will describe evidence that imidazole propionate forms a double spot in the solvent *tert.*-butanol–acetone–formic acid–water (160:160:1:39), using Whatman No. 1 filter paper. The double spot is avoided by the use of acid-washed paper. Investigators may waste time and effort if they are not aware of the distinctive double spot formed by this imidazole. The phenomenon of multiple zone formation has been reviewed and a theoretical treatment has been presented by KELLER AND GIDDINGS[6].

Methods

Ascending chromatography was carried out on Whatman No. 1 filter paper. Solvents were commercial analytical reagent grade. Chromatograms were formed into cylinders and developed 23–26 cm from origin at 25–30°. Imidazole propionate was obtained from Calbiochem (m.p. 207–208°), Koch-Light Laboratories (m.p. 208–209°) and by hydrogenation of urocanic acid with palladium catalyst (m.p. 210–211°) as described by KRAML AND BOUTHILLIER[7]. It was recrystallized from water–ethanol–acetone (1:1:1). The compound was dissolved in deionized water for chromatography unless additions are stated. After drying, the paper was sprayed with diazotized sulfanilic acid. Imidazolepropionic acid was determined as described by TABOR[8].

Results and discussion

The double spot produced by imidazolepropionic acid is shown in Fig. 1. The two spots are identical in color. The double spot occurred when either of the two commercial products were used. When the compound was synthesized in our laboratory, both spots appeared and increased together as the synthesis proceeded. The spots are clearly and significantly separated, and are joined by a diffuse area of color as described by KELLER AND GIDDINGS[6]. The faster spot contains 10 % of the total as determined after elution. The R_F values for the two spots are 0.35 and 0.23. The faster and smaller spot corresponds to the value of 0.36 given in the literature[1,3]. The double spot was also found when imidazole propionate was added to urine and the mixture chromatographed and when descending chromatography was employed.

The R_F values of several imidazoles were found to be histidine, 0.00; urocanic acid, 0.58; imidazolelactic acid, 0.05; imidazoleacetic acid, 0.05; and histamine, 0.03. Imidazolepropionic acid is the only imidazole tested which gave a double spot in this solvent. Other imidazoles which might be contaminants do not correspond to either of the R_F values for the double spot.

The pH of the imidazolepropionic acid placed at the origin has a marked effect on the occurrence of multiple spots. The pH of the solution was altered by addition of NaOH and HCl. At pH 12 a bottom spot (R_F 0.04) predominates with a middle spot leading and touching the intense bottom spot. As pH is gradually lowered the middle spot predominates (R_F 0.16 to 0.24) and becomes compact and the bottom spot fades out. At the same time a trace of the upper spot begins to appear (R_F 0.3 to 0.35), until at pH 1 a single spot is observed (R_F 0.30 to 0.35).

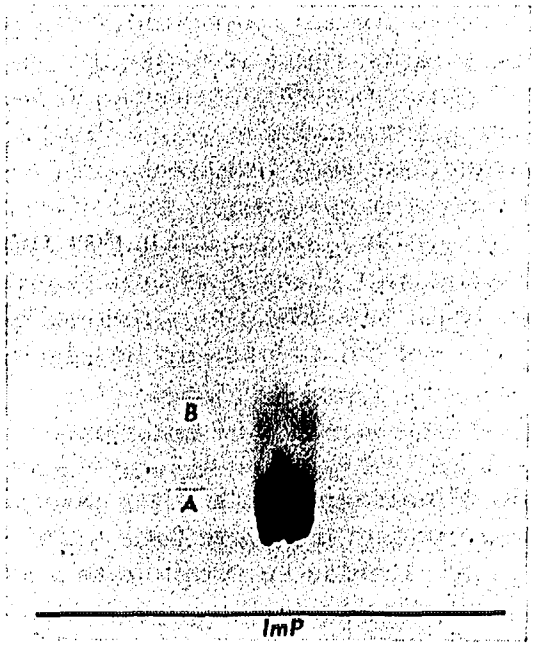

Fig. 1. Double spot formation by imidazolepropionic acid; 0.4 μmole applied to Whatman No. 1 paper; solvent: *tert.*-butanol–acetone–formic acid–water (160:160:1:39); spray: diazotized sulfanilic acid.

Evidence that the formic acid content of the solvent system affected the multiple spot formation was obtained. As the formic acid is increased from 0 to 20 ml (water content adjusted accordingly) the apparent quantity of imidazolepropionic acid in the slow spot changes from 100 % to 0 % as more and more of the compound appears in the fast spot.

In a bidimensional chromatogram using the same solvent in both dimensions one expects four spots if there are two forms of the compound in equilibrium, and two spots if two separate compounds are present. However, three spots were found. On the first pass the imidazole propionate produces two spots. On the second pass, the slower spot (A) splits again into two spots (A and B), but the faster spot (B) remained as one discrete spot, and none of the slower spot could be detected. The slower substance (A) is converted by a slow reaction to the faster substance (B), but after the conversion B did not revert to A on the second pass. To examine this further, two bands (A and B) from a chromatogram were eluted by descending chromatography with water, concentrated under reduced pressure and each rechromatographed. The lower band (A) broke up into two spots when rerun. However, the upper band gave only one discrete fast spot. It appears that imidazolepropionic acid (A) is converted to a new compound (B) by development of the chromatgram. This type of result is discussed by KELLER

AND GIDDINGS[6]. This new compound (B) was stable and was not reconverted to the original (A) during rechromatography or by altering pH. It is not likely, therefore, that we were dealing with two ionic species of the same compound.

It was possible to prevent double-spot formation by washing the paper in 2 *N* acetic acid followed by a rinse in deionized water. Commercially acid-washed papers also were effective, including Whatman No. 40, Schleicher & Schüll (S and S) 589 Black and 589 White. In order to determine if inhibition of double-spot formation was due to acidification of the paper, Whatman No. 1 was washed in deionized water. A single wash prevented double-spot formation, although considerable "heading" of the spot was observed. Whatman No. 1 paper from another package obtained from another laboratory also gave distinctive double spots, as did an unwashed S and S paper, 2041. HANES AND ISHERWOOD[9] working with inorganic phosphate attributed ghost spots to calcium and magnesium ions in the paper, and found that acid washing of the paper eliminated multiple spots. In order to see if the addition of ions at the origin of washed-paper chromatograms would restore double-spot formation, we placed the following inorganic compounds at separate origins at two concentrations (0.02 μmole and 0.05 μmole): $MgCl_2$, $CaCl_2$, KCl, NaCl, $CuCl_2$, $ZnSO_4$. Imidazole-propionic acid (0.1 μmole) was also placed at the origins. No double spots occurred. In a similar experiment with unwashed paper, inorganic ions did not change the double-spot formation.

The conversion of A to B is finite. The reverse reaction is slow or does not occur. A and B have different mobilities which are affected by the pH of the applied solution and by the formic acid concentration in the chromatographic solvent. Chromatography of A produces two spots and chromatography of B produces one spot whether done by elution or by two dimensional chromatography. An unknown component of the paper is responsible for the reaction since washing the paper prevents the double spot. Other imidazoles either do not undergo the reaction or the two forms have the same mobility.

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