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1 D. EMRICH, H. REICHENBACH, G. HOFFMANN AND W. KEIDERLING, *Nucl.-Med.*, 6 (1967) 149.
2 E. M. VOLPERT, M. MARTÍNEZ AND J. OPPENHEIMER, *J. Clin. Endocrinol. Metab.*, 27 (1967) 421.
3 R. GMELIN AND A. J. VIRTANEN, *Acta Chem. Scand.*, 13 (1959) 1469.
4 E. ZAPPI, *J. Chromatog.*, 31 (1967) 241.
5 E. ZAPPI, to be published.
6 E. ZAPPI, *Dissertation*, Freie Universität Berlin (1967).
7 S. C. WERNER AND R. J. BLOCK, *Nature*, 183 (1959) 406.
8 H. WEINERT, H. MASUI, I. RADICHEVICH AND S. C. WERNER, *J. Clin. Invest.*, 46 (1967) 1264.
9 E. ZAPPI AND G. HOPPE, *Z. Klin. Chem.*, 5 (1967) 209.
10 E. ZAPPI AND G. HOPPE, *Nucl.-Med.*, 6 (1967) 420.

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Separation of imidazoles in the histidine-loading test

The histidine-loading test, originally devised by LUHBY *et al.*[1], has been used clinically for about ten years now, mainly for the detection of deficiency of folic acid and/or vitamin B_{12}. Such a deficiency is shown by the appearance of urocanic acid[2] and/or formiminoglutamic acid (FIGLU) in the urine after an oral load of 15 g L-histidine monohydrochloride.

The screening of urine for these two metabolites was facilitated when ROBERTS AND MOHAMED[3] introduced a thin-layer chromatographic separation on cellulose, with an *n*-butanol–acetic acid–water solvent. In the same year, MIDDLETON[4], using two-dimensional paper chromatography, showed the importance of examining other histidine metabolites, especially other imidazoles.

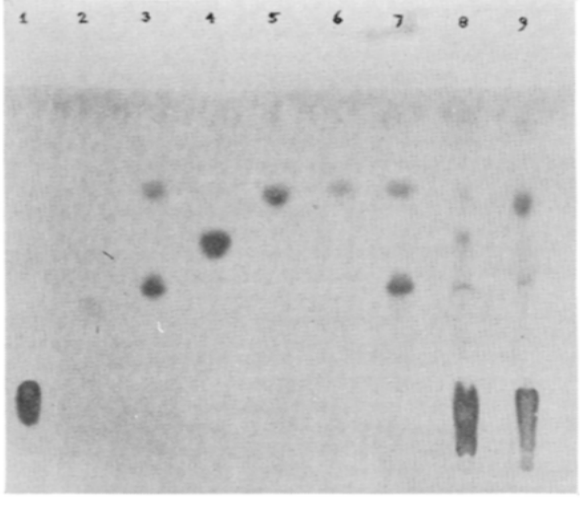
From a technical point of view, there are two problems in the use of *n*-butanol–acetic acid–water as solvent. In the first place, as is well known, esterification occurs readily between the butanol and acetic acid, and the solvent gives a good separation of imidazoles only when freshly prepared. Secondly, especially in pregnancy cases where altered kidney function allows large quantities of histidine to appear in the urine, the chromatography consists of separating small quantities of metabolites in the presence of large amounts of histidine. This, in turn, gives rise to streaking of histidine, often reaching up to the spot of imidazolelactic acid.

We find that chromatography on thin layers of cellulose, with the solvent isopropanol–formic acid–water (40:2:10), as used by JONES AND HEATHCOTE[5] for amino acid chromatography, gives very good separation of imidazoles and also allows the application of quite large amounts of histidine. This is because the double spot of histidine does not migrate far from the origin, whereas the R_F values of other imidazoles are relatively high. Typical R_F values obtained for a number of imidazoles are given in Table I.

TABLE I

R_F VALUES OF IMIDAZOLES

Imidazole	R_F
Histidine	0.19
Imidazolecarboxylic acid	0.41
Imidazolelactic acid	0.47
Imidazoleacetic acid	0.57
Imidazolepropionic acid	0.65
Imidazoleacrylic acid (urocanic acid)	0.68



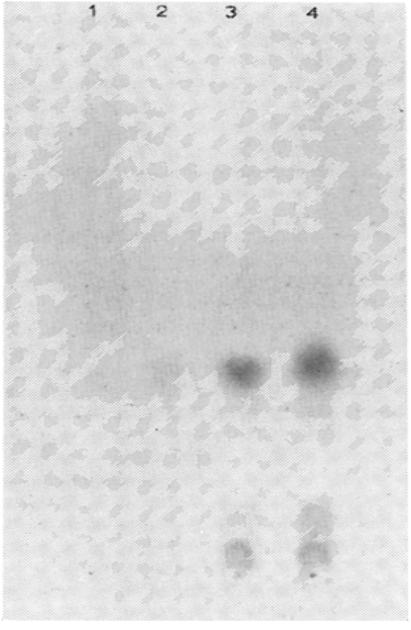


Fig. 1. Chromatography of imidazoles on cellulose thin-layer, 250 μ thick. Solvent, isopropanol–formic acid–water (40:2:10); spray reagent, Pauly[6]. 1 = Histidine. 2 = Imidazolecarboxylic acid. 3 = Mixture of imidazolelactic acid (lower spot) and urocanic acid (upper spot). 4 = Imidazoleacetic acid. 5 = Imidazolepropionic acid. 6 = Urocanic acid. 7 = Mixture of 2 and 3, showing sufficient separation of imidazolecarboxylic acid and imidazolelactic acid for identification, especially since the former gives a yellow and the latter a red colour. 8 = Human urine after histidine loading (showing, from bottom to top, large zone of histidine, with small spots of imidazolelactic acid, imidazoleacetic acid and urocanic acid). 9 = Rat urine after histidine loading (showing histidine, imidazolelactic acid, faint imidazoleacetic acid and prominent imidazolepropionic acid spots).

Fig. 2. Chromatography of urine in the histidine-loading test, to show imidazolepyruvic acid. Conditions as in Fig. 1. Spray reagent: 0.5 % $FeCl_3$ in ethanol. 1 = Before histidine loading. 2 = 2 1/2 hours after loading. 3 = 5 hours after loading. 4 = Synthetic imidazolepyruvic acid added to sample 3 to show chromatographic identity of spots.

Because of the close proximity of imidazolepropionic acid to urocanic acid, it is not easy to distinguish between the two except by the difference in colour obtained with the Pauly reagent (red and brown, respectively). However for a screening test, this is unimportant, since MIDDLETON[4] has shown that either of these metabolites gives a strong indication of folic acid and/or vitamin B_{12} deficiency.

Another substance that we find quite frequently in pregnancy cases is imidazolepyruvic acid, the representative of the transamination pathway of histidine metabolism. This has an R_F value similar to imidazolecarboxylic acid, and tends to streak somewhat below imidazolelactic acid. However, it may be detected as a blue spot when a separate plate is sprayed with an alcoholic solution of ferric chloride (0.5%, w/v). The reaction is intensified by warming with a hot-air blower.

Fig. 1 shows the separation of imidazoles in human and rat urines after histidine loading, compared with standards. Staining was performed with the Pauly reagent[6]. Fig. 2 shows imidazolepyruvic acid in urine after histidine loading, visualised with ferric chloride spray. For comparison more synthetic imidazolepyruvic acid has been added to the same urine; this is necessary because the substance runs with a higher R_F from a pure aqueous solution.

Chromatography in the isopropanol–formic acid–water system is now a routine procedure in our laboratory for the assessment of histidine-loading tests, and the same solvent may be used repeatedly for an indefinite period.

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1 A. L. LUHBY, J. M. COOPERMAN AND D. N. TELLER, *Proc. Soc. Exptl. Biol. Med.*, 101 (1958) 351.
2 M. C. BENNETT AND I. CHANARIN, *Nature*, 196 (1962) 271.
3 M. ROBERTS AND S. D. MOHAMED, *J. Clin. Pathol.*, 18 (1965) 214.
4 J. E. MIDDLETON, *J. Clin. Pathol.*, 18 (1965) 605.
5 K. JONES AND J. G. HEATHCOTE, *J. Chromatog.*, 24 (1966) 106.
6 I. SMITH AND M. BIRCHENOUGH, in I. SMITH (Editor), *Chromatographic Techniques*, Heinemann, London, 1958, p. 145.

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