CHROM. 5095

Separation of phenolic and indolic acids from untreated urine

The author has described methods[1-4] for chromatographing crude or unprocessed samples. These have been based on the following principles:

(1) Using multiple runs, it is possible to get compact spots, even in the presence of contaminants. The routine type of multiple run, however, does not favour the separation of substances having high R_F values[5]. This drawback is overcome by using multiple runs of increasing running times[4]. Selection of the solvent is important as the influence of the contaminants in distorting the separations varies with the solvent.

(2) In the case of aromatic, heterocyclic and less polar aliphatic compounds, it is possible to find solvents which will separate these compounds well from water-soluble contaminants. Once separated, the compounds are not influenced by the contaminants in subsequent runs[1,2]. If the spots are elongated in the first run, another run in the same direction results extremely well in compact spots[2].

(3) If a sample is applied on the paper in the form of a large rectangular spot, the concentration of the contaminants per unit area is rather small and the compounds may separate out as compact bands during a run. These bands can be made to form compacted spots before the actual two-dimensional separation[3].

The method described in this paper also demonstrates the practicability of the principles outlined above. In this method, urine corresponding to 0.8 mg of creatinine is applied, as such, on Whatman 3MM paper for chromatographic separation of phenolic and indolic acids, avoiding the entire extraction step.

Experimental

Urine corresponding to 0.8 mg of creatinine is applied as a 5.5 × 1.0 cm rectangular spot in one corner of a sheet (34 × 28 cm) of filter paper (Whatman 3MM). The 5.5-cm long edge of the spot lies parallel to the 34-cm long edge of the paper and 1.5 cm distant from it. The 1-cm long edge of the spot is 2.5 cm away from the 28-cm long edge of the paper.

In order to spread out phenolic and indolic acids from the rectangular spot, 3 consecutive ascending runs are employed along the breadth of the paper in the solvent system ether–xylene–formic acid–methanol–water (600:200:80:10:6). In each of the 3 runs, the solvent rises (in 10 min) to a line drawn 8 cm from and parallel to the upper edge of the rectangular spot. This helps to separate phenolic and indolic acids as compact bands from aliphatics and salts which remain behind in the rectangular spot.

Compacting the bands into spots. For phenolic acid chromatograms, compacting the bands into spots is done using 95% ethanol in an ascending run (45 min) in an uncovered chromatographic chamber placed under a fan. The run is made along the length of the paper, after cutting away the spot rectangle from the paper. The solvent front slowly advances to and stays in line with the upper 1-cm edge of the rectangular hole. For this run, an all-glass chamber (40 (height) × 21 × 16 cm) is being used in this laboratory. A petri dish containing 50 ml of 95% ethanol is placed 28 cm above the bottom of the chamber. Ordinary filter paper is supported along the walls of the chamber and is immersed in ethanol which keeps the filter paper soaked as well as ensures that the chamber is saturated with vapour between the compacting runs. This

prevents (during the compacting run) too much evaporation of alcohol from the solvent in the dish or from that ascending on the paper. Use of the fan may be required to stop the ascent of solvent at the desired height.

For indolic acids, the solvent system ethyl methyl ketone–pyridine–water (25:5:20) is used instead of 95% ethanol. The migration of this solvent cannot be stopped at the desired height under the conditions mentioned above, but the rate of ascent can be reduced considerably by using the fan. 20 to 30 min may be needed

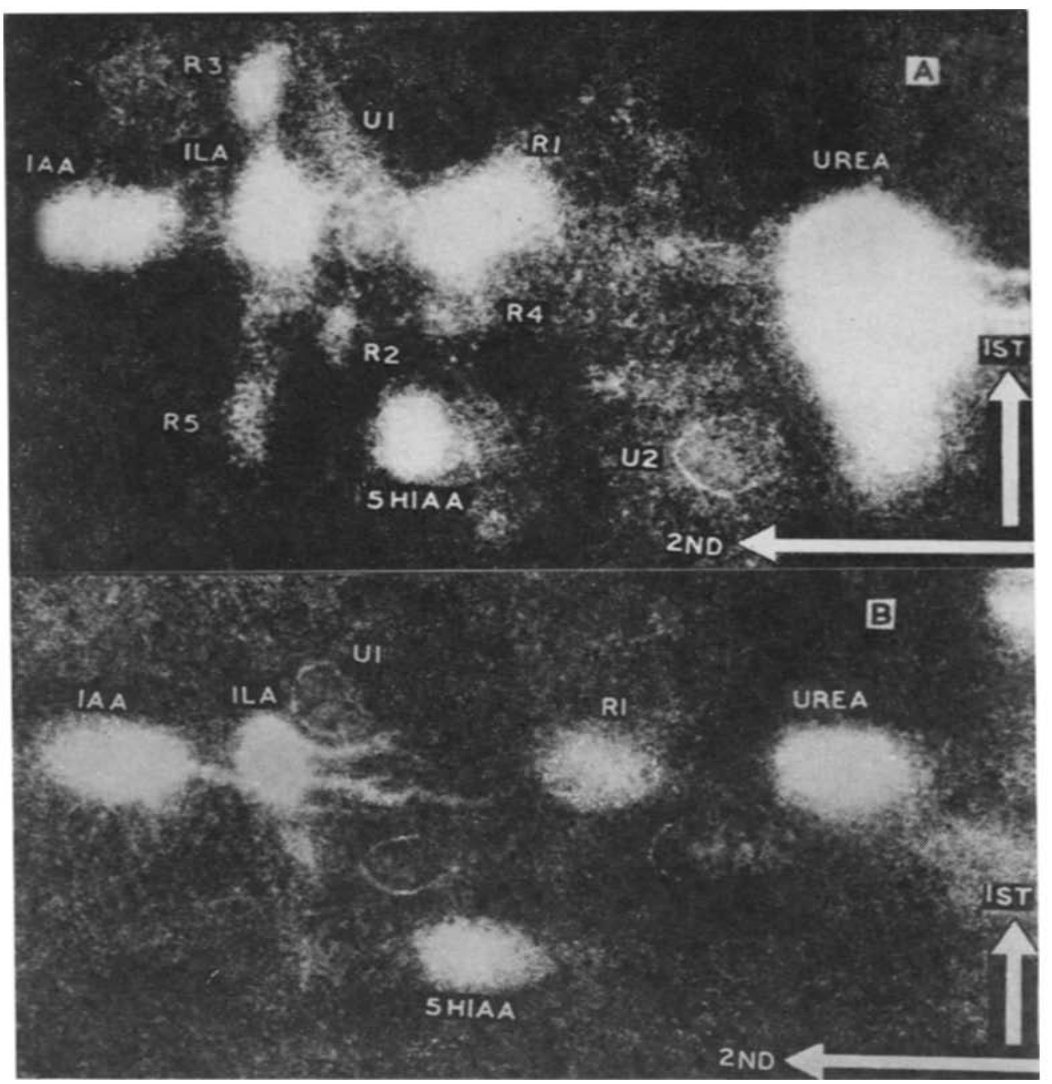

Fig. 1. Segments of two-dimensional (ascending) chromatograms for urinary indolic acids on Whatman 3MM paper. (A) Chromatogram prepared according to the present method by spotting urine corresponding to 0.8 mg of creatinine, as such, on paper. (B) Chromatogram from extract[6] (same extract as for phenolic acids), corresponding to 0.8 mg of creatinine, of the same urine sample as used for the above chromatogram. Ehrlich reagent is used for staining the two chromatograms. The solvent systems are isopropyl alcohol–*n*-butyl alcohol–*tert.*-butyl alcohol–water–ammonia (4:2:2:2:1) and ether–xylene–formic acid (85%)–methanol–water (500:300:80:10:6), for the first and the second runs, respectively. Abbreviations for spots are as follows: IAA = indolylacetic acid; ILA = indolyllactic acid; 5 HIAA = 5-hydroxyindolylacetic acid; U1 and U2 = unidentified indolic compounds (stain purple-blue with Ehrlich reagent); R1, R2, R3, R4, R5 = unidentified compounds (stain red with Ehrlich reagent).

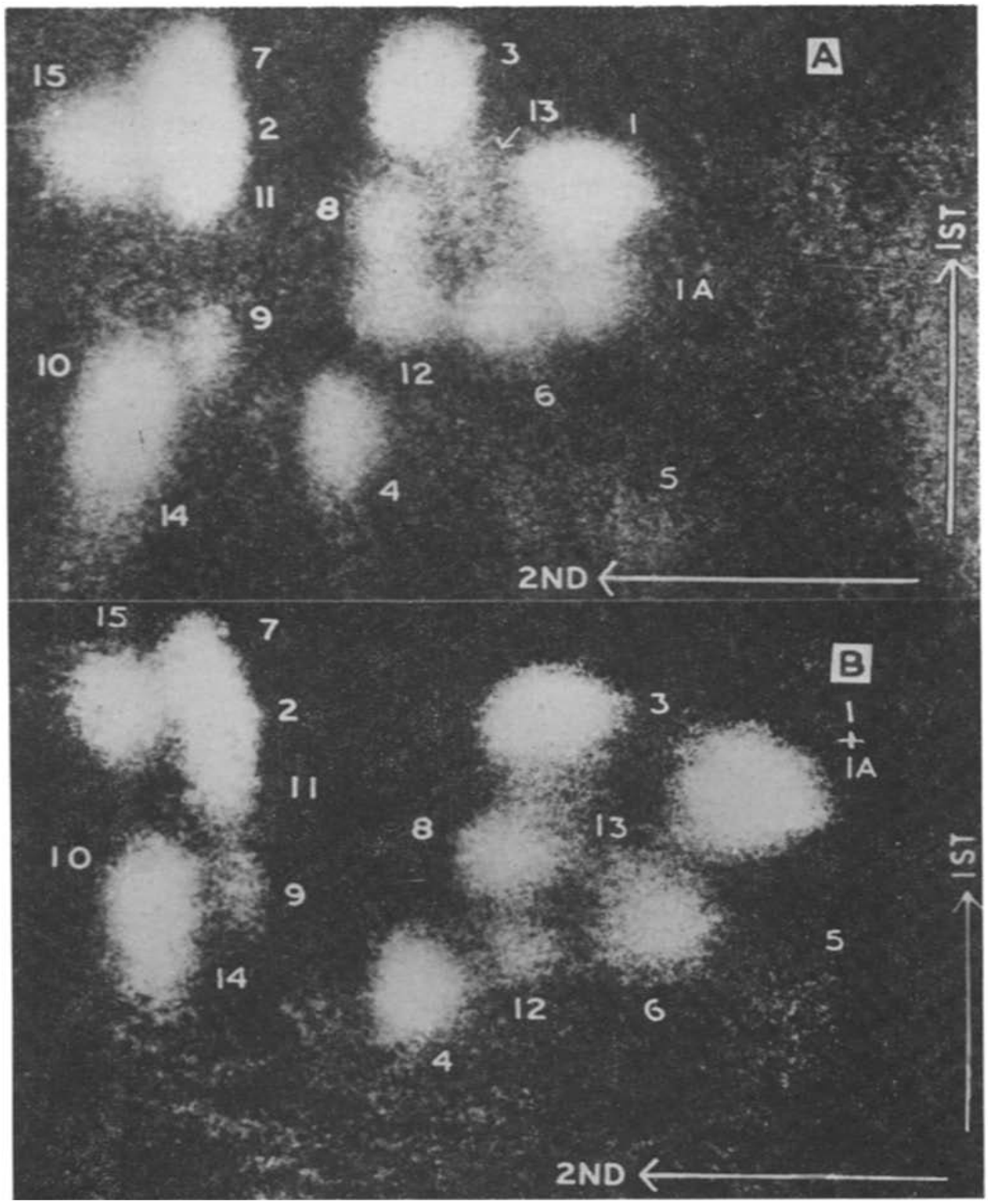

Fig. 2. Segments of two-dimensional (ascending) chromatograms for urinary phenolic acids on Whatman 3MM paper. (A) Chromatogram prepared according to the present method by spotting urine corresponding to 0.8 mg of creatinine, as such, on paper. (B) Chromatogram for extract[a] corresponding to 0.8 mg of creatinine, of the same urine sample as used for the above chromatogram. *p*-Nitroaniline reagent is used to stain the two chromatograms. Solvents are isopropyl alcohol–*n*-butyl alcohol–*tert.*-butyl alcohol–water–ammonia (4:2:2:2:1) and ether–xylene–formic acid (85%)–methanol–water (500:300:80:10:6), for the first and the second runs, respectively. Numbers indicate phenolic acid as follows: 1 = *m*-hydroxyhippuric acid; 1 A = unidentified spot (stains red); 2 = *p*-hydroxyphenylacetic acid; 3 = *m*-hydroxyphenylhydracrylic acid; 4 = unidentified spot (stains orange-yellow); 5 = *p*-hydroxyhippuric acid; 6 = 3-methoxy-4-hydroxymandelic acid; 7 = *m*-hydroxyphenylacetic acid; 8 = unidentified spot (stains orange-yellow); 9 = *o*-hydroxyhippuric acid; 10 = *p*-hydroxybenzoic acid; 11 = homovanillic acid; 12 = 5-hydroxyindolylacetic acid; 13 = 3-methoxy-4-hydroxyphenylhydracrylic acid; 14 = vanillic acid; 15 = *m*-hydroxybenzoic acid.

for the solvent to ascend to the desired height. In this case, therefore, two (30 min each) or three (20 min each) consecutive runs are needed to compact indolic acid bands into spots.

Runs for separating the spots. The paper is then subjected to 2 consecutive runs (1 h, 15 h) in the solvent system isopropyl alcohol–*n*-butyl alcohol–*tert.*-butyl alcohol–water–ammonia (4:2:2:2:1), in the same direction as that of the compacting run. Before these runs, it is advisable to cut away a parallel strip of the paper 5 cm from the lower edge. The lower segment, not containing phenolic or indolic acids, is discarded.

The final run (3 h) in the solvent system ether–xylene–formic acid (85%)–methanol–water (500:300:80:10:6), is at right angles to the direction of the latter run.

Phenolic and indolic acids are revealed by staining the chromatograms with *p*-nitraniline and Ehrlich reagent, respectively[6].

Results and discussion

Good separations obtained by the present method (see Figs. 1 and 2) demonstrate the practicability of the principles discussed in the Introductory section. These principles have thus been used for chromatography of amino acids[1,3,4], phenolic amines[2], phenolic acids and indolic acids, and the scope of application to other fields is ample in separations both on paper and on thin layers.

Recoveries of different phenolic and indolic acids in the present procedure are comparable to those obtained in the conventional procedures (Figs. 1 and 2). When indolic acid chromatograms are stained with the Ehrlich reagent, some unidentified red spots are seen besides the known indolic acid spots[7]. These red spots are more abundant in chromatograms obtained by the present method. Phenolic acid chromatograms obtained by the present procedure, when compared with the conventional ones (prepared by chromatographing an extract[6]), the following features are noted:

(1) By the present method there is better separation of spots in the first migration (Fig. 2). The spot of *p*-hydroxyhippuric acid is well separated from other spots and the spot of 3-methoxy-4-hydroxyphenylhydracrylic acid is well separated from that of 3-methoxy-4-hydroxymandelic acid.

(2) An unidentified spot (1 A in Fig. 2) is well separated from the spot of *m*-hydroxy hippuric acid. The two spots overlap in the conventional chromatograms.

This study is partly supported by a research grant from the Indian Council of Medical Research. Mr. GROVER AND Mr. RAVINDER are thanked for preparing the illustrations in this paper.

Department of Physiology, Medical College, Rohtak (India) A. S. SAINI

1 P. I. SINGH, V. R. BAJAJ AND A. S. SAINI, *J. Chromatog.*, 43 (1969) 554.
2 A. S. SAINI, *J. Chromatog.*, 49 (1970) 349.
3 A. S. SAINI, *Clin. Chim. Acta*, 31 (1971) 479.
4 A. S. SAINI, *Clin. Chim. Acta*, 31 (1971) 482.
5 H. HALPAAP, in I. SMITH (Editor), *Chromatographic and Electrophoretic Techniques*, Heinemann, London, 1969, p. 840.
6 I. SMITH (Editor), *Chromatographic and Electrophoretic Techniques*, Heinemann, London, 1960, pp. 183, 291.
7 M. D. ARMSTRONG, K. N. F. SHAW, M. J. GORTATOWSKI AND H. SINGER, *J. Biol. Chem.*, 232 (1958) 17.

Received September 21st, 1970