CHROM. 5505

A revised method for paper chromatographic study of urinary phenolic amines

This paper describes a revised version of the author's previously reported method[1] for the study of urinary phenolic amines. As compared to the previous method, the present method is simpler, less time-consuming and results in similar recoveries of different phenolic amines. It is also suitable for the study of urinary indolic amines.

Experimental

Hydrolysed[2] urine is adjusted to pH 7.0 (indicator paper) using solid sodium carbonate and a volume corresponding to 120 mg of creatinine is evaporated in the dark to leave behind a semi-solid mass. In this laboratory, evaporation of urine is carried out in a 10.5-cm petri dish placed in a sand bath (sand moistened with an oil) at 40°, placed under a fan, in a dark room. The dish accommodates about 70 ml of urine and therefore the requisite volume (corresponding to 120 mg of creatinine) is evaporated by two additions to the dish. The step takes about 16 h, and could also be carried out in a rotary evaporator.

The semi-solid mass from the dish is transferred to a stoppered 25-ml graduated centrifuge tube with the help of the flattened end of a glass rod. The dish is washed twice with 1 ml of water and the washings are also transferred to the centrifuge tube. With another thin glass rod, the material in the centrifuge tube is reduced to a homogeneous suspension. On centrifugation, the upper aqueous layer (say 3 ml) and the lower layer of solids is formed. Sufficient ethanol (4.5 ml in this case) is added to the tube to give a final concentration of 60 %. This alcohol is not added directly but is first used to wash the dish in which the urine had been dried and washings are transferred to the centrifuge tube. With 60 % ethanol, added similarly, the volume in the centrifuge tube is made up to the 12 or 15 ml mark. The centrifuge tube is vigorously shaken for 5 min and then centrifuged. The great solubility of aromatic amines in 60 % ethanol is well known[2,3].

The volume of the supernatant (X ml) corresponding to about 20 mg of creatinine is transferred to a stoppered 11 × 1.3 cm tube (A), to which is also added $X/8$ ml of borate buffer[4] at pH 10.0, and the final pH is adjusted to 10.0 using 4 N NaOH. Next, 3/2 X ml of washed n-butanol[4] is added to tube A and the contents are vigorously shaken for 10 min. After centrifugation, the upper layer is transferred to another 11 × 1.3 cm stoppered graduated tube (B). The volume of the aqueous layer in tube A is now less than X ml because of removal of ethanol with n-butanol. This butanol–ethanol mixture has greater solubility for aromatic amines as compared to butanol alone and therefore extraction is quantitative into the small volumes used.

To the aqueous layer in tube A, an equal volume of n-butanol–ethanol (3:1) mixture is again added and the contents of the tube are shaken for 5 min. After centrifugation, the upper layer is again added to tube B. If the final volume in tube B is Y ml, then to the contents of tube B are added $Y/2$ ml of borate buffer. This borate buffer[4] is previously saturated with sodium chloride and mixed with 0.5 ml of n-butanol–ethanol (3:1) mixture per 10 ml. The tube is shaken 4 or 5 times to lower

the amount of urea and some other contaminants in the upper organic layer without causing any loss of aromatic amines.

The upper layer from tube B, after centrifugation, is transferred to a stoppered centrifuge tube (25 ml or larger). To this tube, $Y/4$ ml of 0.14 N HCl and 3 Y ml of purified n-heptane[4] are added and the contents are vigorously shaken for 5 min. The lower aqueous layer is the final extract, obtained after centrifugation and discarding the upper layer.

The final extract is not very pure and its volume is about 2 ml. It is, however, easily chromatographed using the principles previously described by the author for paper chromatography of crude extracts[5].

Chromatography of the extract. The extract is applied on a 12 × 1 cm rectangular spot in one corner of a 46 × 40 cm sheet of Whatman 3MM filter paper. The 12 cm edge of the spot lies parallel to the 46 cm edge of the paper and 1 cm distant from it. The 1 cm edge of the spot is 1 cm away from the 40 cm edge of the paper. In applying the extract, care is taken to distribute the extract uniformly over the spot.

Separating the bands of phenolic amines. From the rectangular spot, the aromatic amine bands are separated by three consecutive ascending runs (1 h, 5 h, 16 h) in the solvent[6] isopropanol–ammonia–water (8:1:1) (solvent A), along the width of the paper. Before the third 16-h run, it is advisable to cut away (and discard) a parallel strip of the paper from its lower edge. The width of this strip is related to the solvent front height in the second 5-h run. For the solvent front height of 14 cm, the width of the strip should be 6 cm. The bands of all important aromatic amines will lie above the line of cut since these have R_F values higher[2,6,7] than 0.45 in solvent A. The procedure improves the separation of amine bands in the last 16-h run.

After the last 16-h run, the paper is dried under a fan and cut 1 cm above the solvent front line. Unused paper above the line of cut is discarded. The upper edge of the paper is made lower for the next two ascending runs (1½ h each; the wash runs) in the solvent ether–benzene–formic acid (85 %)–water (700:100:80:7). Aromatic amines do not move in this solvent and the step helps to wash urea and some unwanted aromatic compounds away from the positions of the aromatic amines. After these runs, the paper is freed of formic acid vapour under a fan.

At this time, the urea band is interposed between the aromatic amine bands (on its lower side, considering the paper in position for the above wash runs) and the bands of unwanted aromatic compounds. The segment bearing the unwanted aromatic compounds along with part of the urea band is next removed. For this, the requisite border of the urea band is first identified by carefully staining the paper bit by bit with Ehrlich reagent[6] on the side of the unwanted aromatic compounds. The urea band is usually 2–2.5 cm thick. Thus a cut is made through the urea band 1–1.5 cm away from and parallel to the urea band border revealed above. The segment bearing the unwanted aromatic compounds is discarded. This step for removal of the urea band is apparently based on the fact[2,6,7] that most aromatic amines have R_F values higher than that of urea (0.42) in solvent A.

Compacting the bands into spots. For this the paper is run in a direction at right angles to the direction of the above runs, the solvent ascending along the length of the bands. Before developing, a line is drawn parallel to and at a distance of 3 cm from the lower margin of the paper for this run. The solvent ethanol–ammonia–water (190:5:5) is used for compacting the phenolic amine bands. When the solvent

reaches the line drawn on the paper, the paper is removed from the dish and is dried in air and again run in the same solvent. Before the second run, it is advisable to cut away a parallel strip of the paper 4 cm from the lower edge. The lower segment does not contain any phenolic amines and is discarded. After the compacting runs, the paper is dried under a fan.

The final run (16 h) in the solvent isopropanol–*n*-butanol–isoamyl alcohol–formic acid–water (50:30:20:15:20) (solvent B) is in the direction of the compacting run.

Phenolic amines are revealed by staining the paper with diazotized *p*-nitraniline[6].

Results and discussion

The procedure described in this paper, compared to the author's previous method[1], is simpler and less time-consuming both for the extraction of urinary phenolic amines and for their paper chromatography. Recoveries of different phenolic

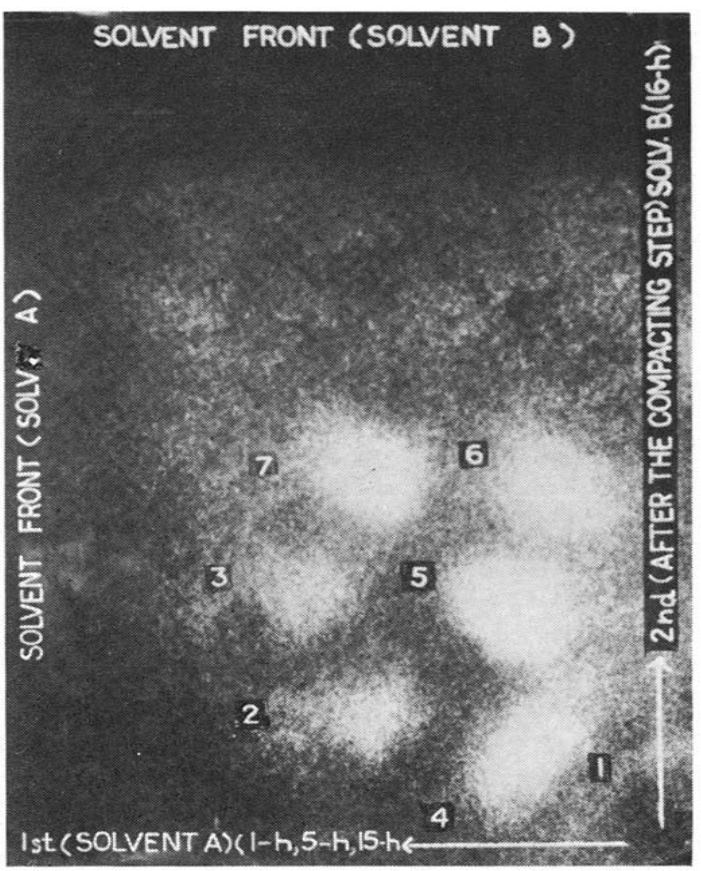

Fig. 1. Segment of a chromatogram for urinary phenolic amines. The extract corresponding to 20 ml of morning urine was spotted on a 12 × 1 cm rectangular spot in one corner of a 46 × 40 cm sheet of Whatman 3MM filter paper and chromatographed as described. *p*-Nitraniline reagent was used to stain the chromatogram. Solvent A: isopropyl alcohol–ammonia–water (8:1:1). Solvent B: isopropyl alcohol–*n*-butanol–isoamyl alcohol–formic acid–water (50:30:20:15:20). Numbers indicate phenolic amines as follows: 1 = normetanephrine; 2 = metanephrine; 3 = *p*-[illegible]e; 4 = unidentified (stains red); 5 = unidentified (stains orange); 6 = unidentified (stains brown); 7 = unidentified (stains red).

amines are similar to those in the author's previous method. A phenolic amine chromatogram (corresponding to 20 ml of morning urine), prepared according to the present procedure, is shown in Fig. 1.

The present method can also be used for the study of indolic amines from (unhydrolysed) urine, but in this case the solvent ethyl methyl ketone–pyridine–ammonia–water (25:5:5:15) has to be used for the step of compacting the bands into spots. In the procedure, recoveries of tryptamine, methoxytryptamine and 5-hydroxytryptamine are 80–90 %, 70–80 % and about 50 %, respectively. Using the method, 5-hydroxytryptamine was always demonstrable in about 20-ml samples of morning urine. One such chromatogram is shown in Fig. 2.

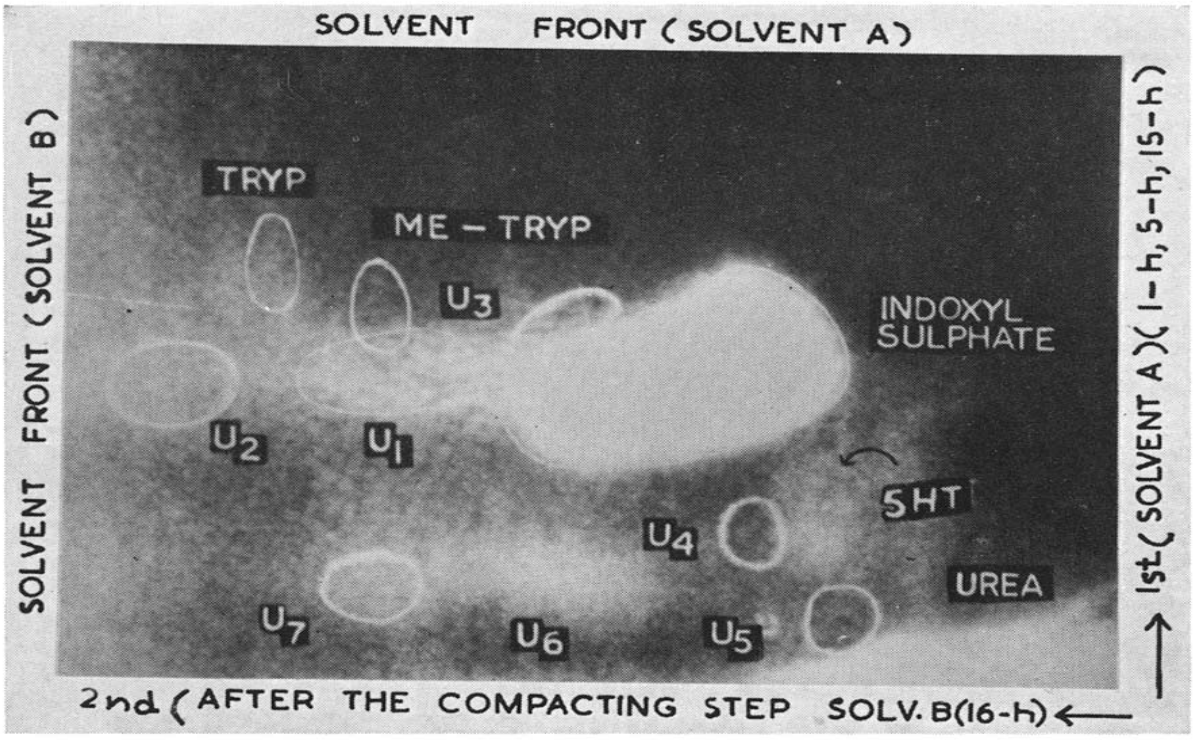

Fig. 2. Segment of a chromatogram for urinary indolic amines. The extract corresponding to 20 ml of morning urine was spotted on a 12 × 1 cm rectangular spot in one corner of a 46 × 40 cm sheet of Whatman 3 MM filter paper and chromatographed as described. Ehrlich reagent was used to stain the chromatogram. Solvent A: isopropyl alcohol–ammonia–water (8:1:1). Solvent B: isopropyl alcohol–*n*-butanol–isoamyl alcohol–formic acid–water (50:30:20:15:20). Abbreviations for spots are as follows: 5HT = 5-hydroxytryptamine; U6 = unknown (stains purple-red). Positions of tryptamine (TRYP), 5-methoxytryptamine (ME-TRYP) and a number of unknown Ehrlich reactors (encountered in the urines studied) are also indicated. U_1 and U_2 are the positions of the spots staining red and yellow, respectively, both appearing late after application of the Ehrlich reagent. The print shown was taken before these two spots had appeared, and what looks like the spot U_1 is actually the slight diffusion of colour from the spot of indoxyl sulphate. U_3, U_4, U_5 and U_7 are the positions of spots staining purple and appearing quickly. In the twelve normal urine samples investigated, the frequency of the above spots was as follows: 5HT, U_1, U_2 (12 samples); U_3, U_4 (4 samples); TRYP, U_5, U_6, U_7 (1 sample); ME-TRYP (no sample).

This study was partly supported by a research grant from the Indian Council of Medical Research.

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First received March 29th, 1971; revised manuscript received June 8th, 1971