CHROM. 4672

## Solvent extraction and paper chromatography of urinary phenolic amines

Ion-exchange methods for extracting urinary phenolic amines are well known[1–4]; but the possibility of a good solvent extraction method for this category of compounds was excluded by KAKIMOTO AND ARMSTRONG[1].

WEISSBACH[5] has used *n*-butanol for the extraction of 5-hydroxytryptamine. In an attempt to adapt this method for the extraction of urinary phenolic amines, the author came across the following difficulties:

The final residue from the urine (corresponding to 20 $\mu$g creatinine) was too bulky to be applied on paper, but by using the first step of the procedure by RODNIGHT[6] it was possible to reduce the bulk without loss of phenolic amines. However, even this extract was too heavy for a good separation and it also contained a number of unwanted phenolic compounds.

The procedure to be described overcomes most of these difficulties and it is possible to obtain a good chromatogram from a urine extract corresponding to 20–25 $\mu$g creatinine. Recoveries of five phenolic amines compare well with those reported for previous ion-exchange procedures.

*Experimental*

Urine is acidified and hydrolysed in an autoclave[2], and then neutralised to pH 5.0 (indicator paper) using NaOH pellets and solid $Na_2CO_3$ and filtered. Borate buffer*, pH 10.0, is added (3 ml buffer for 7 ml filtrate)[5] to the filtrate corresponding to 20–25 $\mu$g creatinine, and the resulting solution ($x$ ml) is then saturated with NaCl and final pH adjusted to 10.0. An equal volume ($x$ ml) of purified *n*-butanol[5] is added to this in a 100 ml (or larger) volumetric flask and the contents are shaken for 10 min. The upper layer is transferred to a 16 × 3 cm tube (A). Another $x$ ml of *n*-butanol are added to the volumetric flask and contents are shaken again for 5 min. The contents of the flask are poured into another 16 × 3 cm tube (B). The upper layer from tube B and the contents of tube A are transferred to the flask originally used for the extraction and 2/3 $x$ ml of borate buffer (pH 10.0, saturated with NaCl and *n*-butanol) are added, and the flask is shaken 4 or 5 times. Part of the contents of the flask are placed in tube A and centrifuged. The upper layer is transferred to a 100-ml beaker. The flask is, then, completely emptied into tube A and after centrifugation the upper layer is again transferred to the 100-ml beaker. 5 ml *n*-butanol used to rinse the extraction flask is also transferred, similarly, to the beaker.

The extract is evaporated to dryness at 40°. The dry residue is taken up in 1 ml (or more) of 60% ethanol which should effect complete solution; the pH of this solution should be between 6 and 7 (indicator paper). To prevent damage to the amines, if pH of this solution is 8.00 or more (the pH of the extract is determined by the temperature at which the extraction with *n*-butanol is carried out), 4 to 5 ml of an 0.02% solution of $KH_2PO_4$ in 90% aqueous methanol should have been added to the butanol extract before it is evaporated. Acetone (11 ml for each ml of 60% ethanol added[6]) is added, in 2 to 3 ml volumes to the ethanolic solution of the residue, mixing after

* The buffer was prepared according to WEISSBACH[5], but the amount of 10 *N* NaOH used was only just sufficient.

each addition. The acetone extract is filtered into a 18 ml weighing bottle using a small funnel. It is advisable to carry out this step as far as possible in an enclosed tank, *e.g.* a chromatography tank, saturated with vapour from an acetone–ethanol–water (12:2:1) mixture. The residue in the beaker is once again washed with 5 ml of acetone containing 1% ethanol. The filtrate in the weighing bottle was evaporated to dryness in a small vessel, *e.g.*, a test tube 3 cm in diameter is cut 2 cm from its base and the lower segment is used for the evaporation.

*Chromatography of the sample*

The residue is taken up in three successive 100-$\mu$l volumes of 95% ethanol and spotted in one corner of a 35 × 35 cm sheet of Whatman No. 1 filter paper, shown as ABCD in Fig. 1. The figure illustrates the method of chromatographing the extract, which is spotted as a 2.0 × 1.5 cm spot, centre at point O. Chromatography is carried out in the direction BA (ascending) for 15 h in isopropyl alcohol–ammonia–water (8:1:1) (ref. 1) (solvent A), keeping BC as the lower edge. During this run, a visible streak STRU, starting from the spot and ending at the solvent front, is produced on the paper. The lower one fifth of this streak does not contain any phenolic amines and can safely be removed to reduce the size of the paper for subsequent runs, by cutting along GH.

Next, the paper is subjected to a short run (designated as the wash run) to remove a number of aromatic compounds from the streak STRU, without disturbing the phenolic amines. For this, a strip of filter paper (length AG and breadth 15 cm) is tacked along AG, with closely set stitches. In order to reduce the distance between the

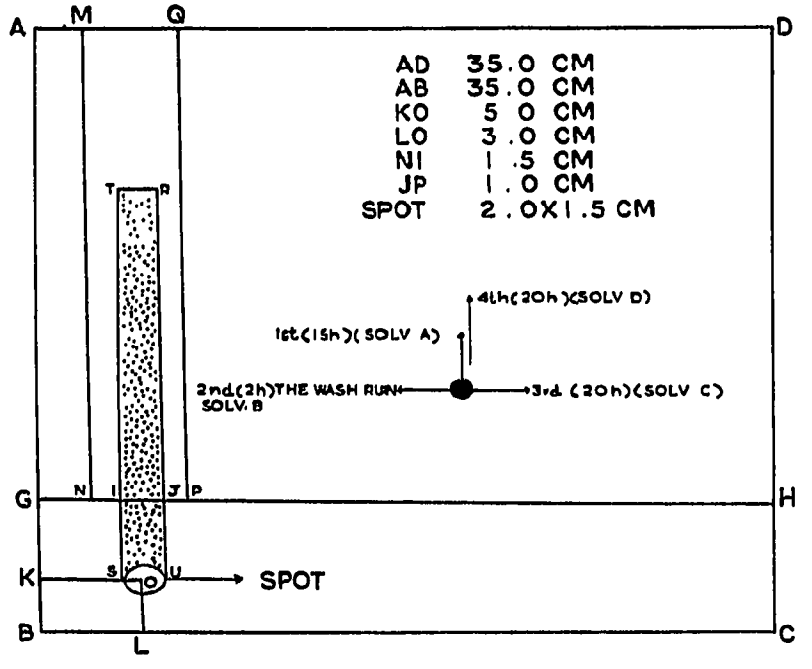


Fig. 1. Diagram illustrating the method of chromatographing the crude extract of phenolic amines. Solvent A = isopropyl alcohol–ammonia–water (8:1:1); solvent B = ether–benzene–formic acid (85%)–water (70:10·8:0.7); solvent C = isopropyl alcohol–*n*-butyl alcohol–isoamyl alcohol–formic acid–water (50:30:20:15.20); solvent D = isopropyl alcohol–*n*-butyl alcohol–*tert.*-butyl alcohol–diethylamine–water (4·2:2:1:2).

streak STRU and level of the solvent in the petri dish, for the ascending wash run, the paper is folded bringing HD on to PQ, and the double flap thus formed is again folded in the middle and the four layers of the filter paper are then stitched along PQ by six well-spaced stitches. This multi-layered side of the paper is placed in the solvent ether–benzene–formic acid (85%)–water (70:10:8:0.7) (solvent B), for the ascending wash run (2 h). The unwanted substances are washed away as streaks on the paper attached along AG, but may be obtained as discrete spots if a slightly different procedure and solvent[7] are used.

After the wash run, the paper is unfolded and cut along MN, 1.5 cm from the edge of the streak STRU, and the paper attached along AG is discarded. MN is now the lower edge of the paper for the next overnight run (20 h, in the direction MD), in the solvent mixture isopropyl alcohol–*n*-butyl alcohol–isoamyl alcohol–formic acid–water (5:3:2:1.5:2) (solvent C). The system *n*-butanol–acetic acid–water (12:3:5)[1], gives

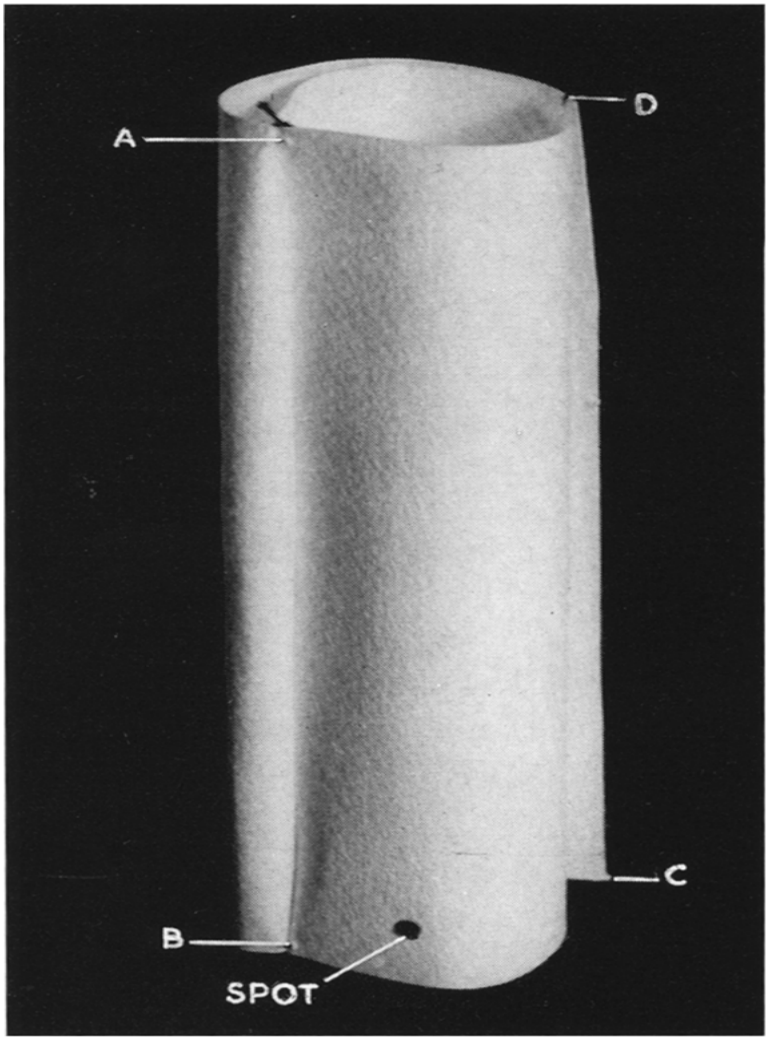


Fig. 2. The method of folding the paper for the first run. Starting with edge AB the paper is folded like a letter S, first in one direction and then in the other. The first fold is held both at its upper and lower ends, by stitches (seen in the illustration), while the second fold is held only by one stitch at the upper end (near end D of the paper). Substances migrate in a perfectly straight line when such a paper is used.

almost similar results if used instead of solvent C. After this, the paper is cut 2 in. below the solvent front line and along PQ (or 1 in. beyond it). HP is the lower edge of the paper for the final overnight run (20 h) in solvent A or in another similar solvent such as isopropyl alcohol–*n*-butyl alcohol–*tert.*-butyl alcohol–water–diethylamine (4:2:2:2:1) (solvent D). The paper is stained with *p*-nitraniline reagent[1] for studying the phenolic amine spots.

For the first ascending run, if the paper is folded as shown in Fig. 2, a 9 cm diameter petri dish will easily accommodate the 35 × 35 cm sheet of filter paper.

*Results and discussion*

Recoveries of *p*-tyramine, methoxytyramine, metanephrine, normetanephrine and octopamine, range between 50 to 80%, in the extraction procedure described. In the ion-exchange methods[1,2], the recoveries for the different aromatic amines lie between 60 to 90%. In our hands, recoveries with the isoamyl alcohol extraction procedure of SANDLER AND RUTHVEN[4] are lower by more than 20% as compared to those obtained by the present method.

In these recovery experiments 10 to 15 $\mu$g of each of the five phenolic amines are added to a borate buffer–water mixture (3:7) saturated with NaCl, which was then adjusted to pH 10.0. The phenolic amines are added to 20 ml and 2 ml of buffer solution, respectively, for the present procedure and for the method of SANDLER AND RUTHVEN[4]. The phenolic amines are extracted according to the respective procedures from these solutions, and the final extracts chromatographed. A standard paper is put up at the same time. After staining the papers with *p*-nitraniline reagent, the spots are quantitatively evaluated by eluting the spots (ref. 4, p. 741) with alkaline methanol and reading the coloured solutions of the metanephrine, normetanephrine and methoxytyramine spots at 540 m$\mu$ and those from *p*-tyramine and octopamine spots at 490 m$\mu$.

Some difficulty may be encountered in spotting the extract, prepared as described in this paper, as it may not dry up easily when applied to the chromatographic paper. However, a good separation is obtained even for a spot which may have failed to dry up completely. The explanation for the good separation obtained in the procedure could be as follows. The first run (solvent A) separates phenolic amines as elongated spots because of the effect of contaminants. It, however, helps to remove phenolic amines away from the influence of the contaminants for the subsequent runs. The second wash run (solvent B) does not contribute much to compacting the spots. In the third run (solvent C) the spots are separated; their shape is elongated in the direction of the first run but compact in the direction of solvent C (see Fig. 3B). The final run, which is in the direction of the first run and in solvent A, or some other similar solvent (solvent D), helps to compact the spots in that direction (see Fig. 3A). A compacting effect as a result of multiple runs is well known but, in the present situation, the effect appears to be more pronounced. It must, however, be remembered that in the present case, the first run takes place in the presence of contaminants and the next one in their absence, while in a simple case of multiple runs no such difference is present.

Such a procedure has a scope of general applicability to the chromatography of crude samples. For a particular category of compounds, one has to know a solvent which, in the presence of contaminants, will cause comparatively less distortion of the spots and will separate contaminants from the actual compounds. A similar procedure

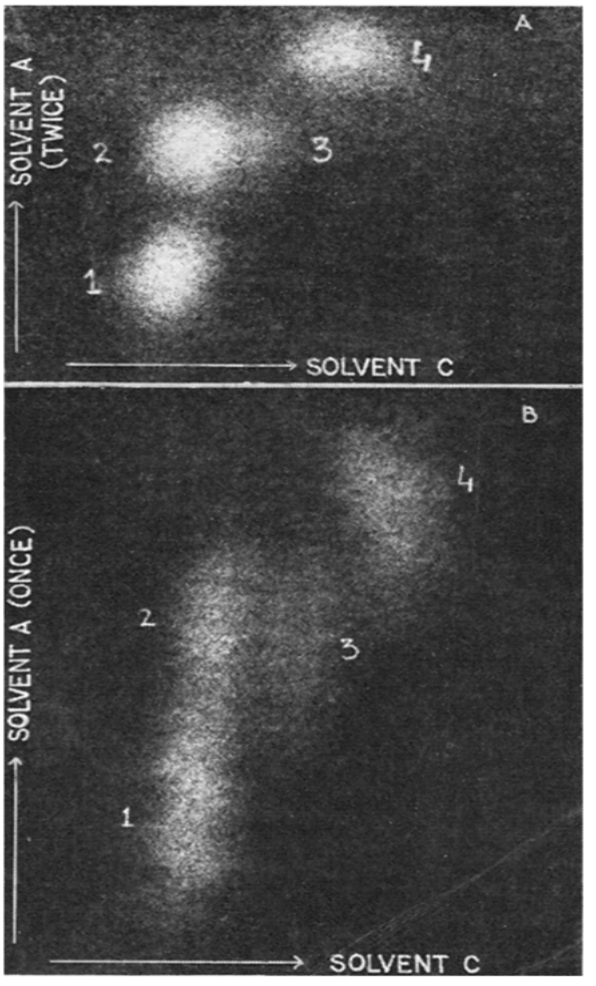


Fig. 3. Chromatograms showing the effect of the final run of the present procedure in compacting the spots. Butanol was shaken with borate buffer pH 10.0, and the residue obtained after evaporating the butanol was spotted on two papers along with a solution of four phenolic amines. 1 = octopamine, 2 = metanephrine, 3 = methoxytyramine, 4 = *P*-tyramine. First run in isopropyl alcohol–ammonia–water (8 1·1) (solvent A) was followed by one (at right angles) in isopropyl alcohol–*n*-butyl alcohol–isoamyl alcohol–water–formic acid (50:30 20·20:15) (solvent C) omitting the wash run of the actual procedure. In paper B, only the above two runs were given while in paper A (as in case of the actual procedure described in this paper) an additional final run was given in the direction of the 1st run using solvent A again.

has already been used for the separation of a particular group of amino acids from untreated urine[8].

The wash run is based on the fact that phenolic and indolic amines have no movement in solvent B or another similar solvent[7]. This is true for *p*-tyramine, methoxytyramine, octopamine, normetanephrine, metanephrine, tryptamine, methoxytryptamine and 5-hydroxytryptamine. Thus, in the wash run, the phenolic amines are not disturbed, whereas a large number of other aromatic compounds which stain with *p*-nitraniline are washed away. In the absence of this run, the known phenolic amine spots in the final chromatograms are overlapped by a number of other *p*-nitroaniline staining spots. However, it should be mentioned that the wash run does not remove some aromatic compounds which are not phenolic amines, since the

final chromatograms contain some *p*-nitraniline staining spots which do not stain with ninhydrin staining reagents[1,2].

Using the extraction and the chromatographic procedure described, the author finds that metanephrine, normetanephrine and *p*-tyramine are always demonstrable in urine samples corresponding to 15–20 $\mu g$ creatinine. SANDLER AND RUTHVEN[4] needed at least 30 ml of urine for such studies.

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

Free gifts of some phenolic amines are gratefully acknowledged from M/s SIGMA (U.S.A.) and M/s CALBIOCHEM (U.S.A.); and Mr. GROVER and Mr. RAVINDER are thanked for preparing the illustrations in this paper.

*Department of Physiology,*
*Rohtak Medical College,*
*Rohtak, Haryana (India)*

A. S. SAINI

1 Y. KAKIMOTO AND M. D. ARMSTRONG, *J. Biol. Chem.*, 237 (1962) 208.
2 T. L. PERRY, K. N. F. SHAW, D. WALKER AND D. REDLICH, *Pediatrics*, 30 (1962) 576.
3 T. L. PERRY AND W. A. SCHROEDER, *J. Chromatog.*, 12 (1963) 358.
4 M. SANDLER AND C. R. J. RUTHVEN, in I. SMITH (Editor), *Chromatographic and Electrophoretic Techniques*, Heinemann, London, 1969, p. 736.
5 H. WEISSBACH, in D. SELIGSON (Editor), *Standard Methods of Clinical Chemistry*, Vol. 4, Academic Press, New York, 1963, p. 197.
6 R. RODNIGHT, *Biochem. J.*, 64 (1956) 621.
7 A. S. SAINI, *Indian J. Med. Res.*, 57 (1969) 370.
8 P. I. SINGH, V. R. BAJAJ AND A. S. SAINI, *J. Chromatog.*, 43 (1969) 554.

First received January 9th, 1970; revised manuscript received February 19th, 1970