A SCREENING TEST FOR PHAEOCHROMOCYTOMA

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Patients suffering from phaeochromocytomata excrete many times the normal amount of noradrenaline in the urine (Euler, 1951). Determination of the excretion of noradrenaline in the urine over 24 hours is thus a valuable aid to the diagnosis of this condition. Few clinical laboratories, however, have the facilities for noradrenaline assay, and there is a need for a simple, reliable, and fairly rapid test to detect the increased secretion of noradrenaline which is characteristic of phaeochromocytoma.

Recently it has been shown (Armstrong and McMillan, 1957; Axelrod, Senoh, and Witkop, 1958) that both adrenaline and noradrenaline are metabolized to 3-methoxy 4-hydroxy mandelic acid or vanillyl mandelic acid. Armstrong and his colleagues showed that in phaeochromocytoma the excretion of vanillyl mandelic acid was much higher than normal. They further showed that, after the tumour's removal, the excretion of vanillyl mandelic acid fell markedly, and they suggested measurement of output of vanillyl mandelic acid as a test for phaeochromocytoma.

In this paper a description is given of a relatively rapid two-dimensional paper chromatographic method for the semi-quantitative measurement of vanillyl mandelic acid in urine.

Method

Urine should be collected over a measured period of time. Although dietary control does not seem to be essential, it might be as well for the subject to avoid bananas (Waalkes, Sjoerdsma, Creveling, Weissbach, and Udenfriend, 1958), coffee, and citrus fruits, which lead to the appearance of spots which might be mistaken for vanillyl mandelic acid. The normal urinary chromatogram (Fig. 1) shows the presence of large amounts of 3-hydroxy-4-methoxyphenylhydric acid, together with a smaller amount of isovanilloyl glycine; these compounds, together with traces of isovanillic and dihydroisoferulic acids, probably owe their presence to the consumption of citrus fruit containing the flavonoid hesperidin (Booth,

Jones, and DeEds, 1958). The subject should also be free from undue stress during the period of collection, since many forms of stress, e.g., exercise, anaesthesia, and surgical operation, can increase the rate of secretion of both adrenaline and noradrenaline.

An ethyl acetate extract of urine (Hill, Ratcliffe, and Smith, 1959) is prepared as follows:

The urine is first acidified to pH 2 (indicator paper) by adding concentrated hydrochloric acid drop by drop, and a known volume (x ml.) equivalent to the amount passed in two minutes is added to ethyl acetate ($5x$ ml.) containing anhydrous sodium sulphate ($2x$ g.). The whole is vigorously shaken and placed in the refrigerator for one hour. During this period it should be shaken occasionally, the cake of sodium sulphate being broken up with a glass rod if necessary.

Chromatography of the resulting extract is carried out by some variant of the method of Armstrong, Shaw, and Wall (1956). It should be appreciated that, apart from the occasional occurrence of large quantities of some constituents, e.g., urea, hippuric acid, salicylic acid, which may tend to lead to distorted chromatograms, successful chromatography is also dependent on atmospheric conditions owing to the volatility of solvents. Factors which favour the production of good chromatograms include the use of a non-volatile second solvent (anisole) and of Whatman No. 20 paper which gives very compact spots; successful development in the first direction may be aided by a relatively high laboratory temperature ($23-25^{\circ}$ C.), and by the choice of tanks which are small or are lined with filter-paper soaked in the solvent used (isopropanol-ammonia) to prevent excessive evaporation of solvent leading to "seizing up" at the solvent front. A certain amount of evaporation is nevertheless desirable, since this results in effectively higher R_F values. It will be evident that optimal conditions may vary from laboratory to laboratory, but the following method always gives chromatograms adequate for screening purposes.

A portion of the ethyl acetate extract (1.25x ml.) is removed and concentrated to a small volume, preferably at a low temperature. It is then transferred quantitatively to a 10 in. square of Whatman No. 20 filter paper, the size of the spot being kept as small as possible; it should be situated about 1 in. from each of two adjacent edges of the paper. Develop-

TABLE I
PHENOLIC ACIDS IN NORMAL URINE

No.	Acid	Trivial Name	Colour with Diazotized p-Nitraniline
1	4-hydroxy-3-methoxy mandelic	Vanillyl mandelic	Violet
2	3-hydroxy-4-methoxy phenyl hydracrylic	—	Blue-violet
3	4-hydroxy-3-methoxy phenyl hydracrylic	—	Violet
4	3-hydroxy-4-methoxy hippuric	Iso vanilloyl glycine	"
5	3-hydroxy-4-methoxy benzoic	Isovanillic	"
6	4-hydroxy-3-methoxy benzoic	Vanillic	"
7	4-hydroxy-3-methoxy phenyl acetic	Homovanillic	Grey
8	3-hydroxy-4-methoxy phenyl propionic	Dihydroisoferulic	Violet
9	4-hydroxy-3-methoxy phenyl propionic	Dihydroferulic	Grey
10	p-hydroxy benzoic	—	Red
11	p-hydroxy phenyl acetic	—	Violet
12	m-hydroxy hippuric	—	Red
13	m-hydroxy phenyl hydracrylic	—	"
14	m-hydroxy phenyl acetic	—	"
15	m-hydroxy phenyl propionic	—	"
16	o-hydroxy hippuric	Salicyluric	"
17	o-hydroxy phenyl acetic	—	Violet-red
18	5-hydroxy indole acetic	—	Red

Substances on the chromatograms which are not yet identified are represented by their colours.

ment of the chromatogram in the first direction (ascending) should proceed overnight, and the faster running direction of the paper should be used. The solvent is isopropanol—5% (w/v) ammonia (4:1). Next day the paper is dried and then run at right angles to the original direction in anisole-glacial acetic acid-water (70:27:3). (It may be necessary to add a few more drops of acetic acid in order to obtain a homogeneous mixture.) Ascending development requires five or six hours. Before running in this solvent it is advisable to "equilibrate" the paper by placing it in a closed vessel containing acetic acid vapour for 30 to 60 minutes. This procedure does not appear markedly to improve chromatograms but minimizes loss of acetic acid from the solvent, which may therefore be used several times before R_F values begin seriously to decrease.

The paper is then allowed to dry in a fume cupboard (at this stage it is impossible completely to remove anisole, which remains absorbed by the paper), sprayed lightly with 10% (w/v) anhydrous sodium carbonate and again dried before phenols are revealed by a final spray with diazotized p-nitraniline (Acheson, Paul, and Tomlinson, 1958): 5 ml. of p-nitraniline (0.2% (w/v) in N-HCl) is diluted with 50 ml. 0.1N-HCl and treated with 1 ml. of 5% (w/v) sodium nitrite. All solutions should be at 0-4° C. After two or three minutes 10 ml. of ice-cold 10% (w/v) sodium carbonate is added and the mixture used immediately. The vanillyl mandelic acid spot is readily distinguished by its position and violet colour. The acid may be estimated by visual

comparison of spots (after chromatography) from 0.5, 1, and 1.5 μ g. of the authentic acid with spots obtained from aliquots of the ethyl acetate extract containing quantities of vanillyl mandelic acid within this range. This refinement is scarcely necessary for the detection of phaeochromocytoma, and comparison may be made against a chromatogram derived from a mixed urine from normal subjects.

Urine specimens have been examined from four proven cases of phaeochromocytoma, from rather more than 100 other patients, most of whom were suffering from essential hypertension, and from 30 apparently normal male subjects, many of whom were investigated on several occasions.

Results

The outstanding feature of the results on the normal subjects and on patients with essential hypertension was their relative constancy, the rates of excretion of vanillyl mandelic acid lying within the range 1-2.5 μ g./min. (values are not corrected for recovery; many chromatograms were larger scale ones, as recommended by Hill *et al.*, 1959). An occasional urine was encountered with a rather higher value, but in each case the patient was under some form of stress.

In contrast the patients with phaeochromocytoma excreted 7.5-11 μ g./min. Visual inspection of the chromatograms was sufficient alone to distinguish these patients from the normal subjects (cf. Figs. 1 and 2).

Urine samples were examined after removal of the tumour in three cases. In two of these there was a marked fall in the excretion of vanillyl mandelic acid. In the third, both symptoms and

FIG. 1.—Chromatogram of urine from normal subject; vanillyl mandelic acid (spot No. 1) = 2 μ g. per minute. For identification of other spots see Table I.

FIG. 2.—Chromatogram of urine from patient with pheochromocytoma; vanillyl mandelic acid (spot No. 1) = 9 μ g. per minute.

a high excretion of vanillyl mandelic acid persisted. The reasons for this are not as yet known.

Discussion

Biological assay of noradrenaline requires facilities for the rat or cat blood pressure techniques which few clinical laboratories possess; fluorimetric assay requires a highly sensitive and costly fluorimeter which again most clinical laboratories do not possess. In contrast, the chromatographic technique for measuring the excretion of vanillyl mandelic acid is simple and requires only apparatus which is standard equipment in the clinical laboratory.

In recent years laboratories where noradrenaline assays are made have become increasingly reluctant to accept urine specimens from other hospitals, since the great majority of specimens prove to be normal. It is suggested that examination of the urine for vanillyl mandelic acid, which can be undertaken by most clinical laboratories, should be used as a screening test. A marked increase in excretion of vanillyl mandelic acid is strong evidence in favour of the diagnosis of pheochromocytoma. Confirmation may then be sought by submitting the specimen for noradrenaline assay.

It should be emphasized that the method recommended in this paper has largely a negative value,

and that, should an apparently positive result be obtained, more detailed chromatographic investigations are essential. In addition, valuable information might be obtained if observations could be made on subjects whose food and drug intakes were known or controlled, for one at least of the above four pheochromocytoma patients excreted unusual quantities of other substances which may be related directly or indirectly to noradrenaline metabolism. Thus excretion of vanillyl mandelic acid (10 μ g./min.) was rather less than that of vanillic acid (13 μ g./min., estimated in urine after acid hydrolysis). The significance of this latter has previously been discussed (Smith, 1958), and the acid may be of considerable interest, for it has been observed in large quantities not only after infusion of noradrenaline but also in adrenalectomized, amongst other, subjects. However, although the absence from these urines of large amounts of vanilloyl glycine strongly suggests that the vanillic acid was not derived from exogenous sources, such observations would clearly be of more value had they been made on patients under strict dietary control. Consumption of vanillin either as sweets, e.g., chocolate, or as medicaments, e.g., laxatives, represents an obvious danger. Also the proximity of isovanilloyl glycine to vanillyl mandelic acid on chromatograms should be noted (Fig. 1), since isovanillin may sometimes be used as a flavouring, although we have no direct evidence to this effect.

Summary

Markedly increased urinary excretions of a noradrenaline metabolite, vanillyl mandelic acid, were found in four cases of pheochromocytoma.

A simple paper chromatographic method for detecting increased amounts of vanillyl mandelic acid in urine is described.

Results confirm the suggestion of Armstrong *et al.* (1956) that the method may be used as a screening test for pheochromocytoma.

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