A NEW URINARY ASSAY FOR SEPARATE NORMETANEPHRINE AND METANEPHRINE WITH APPLICATION FOR THE DIAGNOSIS OF PHAEOCHROMOCYTOMA

ROBERT L. WOLF, CORNELIA E. GHERMAN, JOHN D. LAUER, HAROLD L. FISH AND BURT R. LEVEY

The Mount Sinai Medical Center of the City University of New York

SUMMARY

1. A new, specific and sensitive urinary assay for separate normetanephrine (NM) and separate metanephrine (M) is described.
2. This test employs high-voltage paper electrophoresis after passage of the urine through a resin column.
3. There is no difference in the urinary excretions of either separate NM or separate M in normotensive or hypertensive subjects.
4. Phaeochromocytoma patients excrete significantly larger amounts of separate NM and separate M than normotensive or hypertensive subjects.
5. Incorrect results are occasionally obtained with other urinary assay tests which measure either the combined metanephrines or different urinary catecholamine metabolites for the diagnosis of phaeochromocytoma.

Key words: hypertension, phaeochromocytoma, normetanephrine, metanephrine.

A valid diagnosis of phaeochromocytoma is established by laboratory measurements of the increased urinary excretions of the particular catecholamine metabolites, normetanephrine (NM) and metanephrine (M) (Yoshinaga, Itoh, Ishida, Sato & Wada, 1961a; Yoshinaga Itoh, Ishida, Sato & Wada, 1961b; Smith & Weil-Malherbe, 1962; Taniguchi, Kakimoto & Armstrong, 1964; Pisano, 1960; Brunjes, Wybenga & Johns, 1964; Axelrod, Inscoe, Senoh & Witkop, 1958a; Crout, 1961; Weil-Malherbe, 1964; Bigelow & Weil-Malherbe, 1968; Wolf, Mendlowitz, Roboz & Gitlow, 1964). We have devised a new, specific and sensitive urinary assay for separate NM and separate M and compared the results employing this test with three other tests; two-dimensional paper chromatographic assay of the urine for 3-methoxy-4-hydroxy-mandelic acid (VMA), spectrophotometric assay of the urine for the combined metanephrines and paper chromatoelectrophoretic assay of the urine for the combined metanephrines and 3-methoxy-4-hydroxyphenylglycol (Axelrod et al., 1958a; Wolf, Mendlowitz, Roboz & Gitlow, 1965; Axelrod, Senoh & Witkop, 1958b). The urinary assay for separate NM and separate M is more specific and sensitive than the other tests.

Correspondence: Dr Robert L. Wolf, 20 East 74th Street, New York, N.Y. 10021, U.S.A.
NM and separate M was accomplished by the sequential procedures of urinary hydrolysis, passage through an Amberlite CG-50 resin column, volume reduction by flash evaporation, extraction with ethyl acetate, isolation and separation of separate NM and separate M by high-voltage paper electrophoresis, staining with diazotized p-nitroaniline and scanning, integrating and recording with a densitometer. This sensitive test was performed on urine specimens from sixty-three patients with phaeochromocytoma and over 2000 other subjects. The results indicate that the correct diagnosis was always established with this new assay for separate NM and separate M and also with the chromatoelectrophoretic assay. The other assay techniques occasionally yielded inaccurate results (Wolf, Mendlowitz, Roboz, Naftchi & Gitlow, 1966).

METHODS

Extraction and electrophoresis

For each assay 10 ml of urine was used. The pH was adjusted to 0.9 with 6 M-HCl. After hydrolysis for 20 min in a boiling-water bath, the test material was cooled in ice until room temperature was reached. The pH was adjusted to 6.0-6.5 with 1 M-NaOH, and the volume was diluted to 20 ml with water. Then the sample was passed through a column (5 cm x 1.5 cm) of Amberlite CG-50 resin as described by Pisano (1960) in two 10 ml portions. The column was subsequently washed with 15 ml of water and eluted with 10 ml of aq. 4 M-NH₃. A 300 ml pear-shaped flask was employed to collect the eluate. By employing a rotating flash evaporator the sample was concentrated to approx. 1-2 ml and transferred with a Pasteur pipette, to a 45 ml graduated centrifuge tube. The flask was washed twice with approx. 1 ml of aq. 4 M-NH₃ and the washings were added to the eluate which was extracted with 2 vol. of ethyl acetate by agitation for 3 min with an automatic shaker followed by centrifugation and collection of the top layer in another tube. This extraction was repeated three times and the bottom layer was discarded. (The extract is stable overnight in the refrigerator.) The ethyl acetate extract was blown to dryness in a water bath (temperature less than 50°C) and the dried extract was re-dissolved in 100 µl of ethyl acetate in preparation for electrophoresis (spotting is recommended immediately after the addition of ethyl acetate).

High-voltage electrophoresis was performed with a Savant Instruments Inc. apparatus (Model FP-30 A Flat Plate, HV-5000 A Power Supply and RWC-50 A Recirculating Water Cooler) and Tris buffer, pH 8.4 (36.3 g of Tris in 90 ml of acetone, diluted to 3:1 with water at a pH of 8.4 adjusted with 6 M-HCl). The Whatman 3MM paper strips measured 3.8 cm x 91.4 cm. Spotting was accomplished by means of a capillary tube at the middle of the strip. A hair dryer was used to facilitate drying. The spot diameter was less than 0.5 cm. Three normetanephrine standards were spotted on similar strips (for example, 0.5 µg, 1.0 µg and 1.5 µg of normetanephrine from a stock solution containing 1 µg of normetanephrine per 10 µl of water). Then the strips were immersed in the buffer, to the edge of the spot, and blotted on heavy filter paper. The strips were applied to the high-voltage electrophoresis plate; the spot was at the centre of the plate and the ends were immersed in the buffer vessels. The apparatus was run for 2 h at 3000 V. The strips were then removed and dried in an oven (149°C) for approx. 20 min and stained with diazotized p-nitroaniline (a mixture of 0.1% p-nitroaniline, 0.2% NaNO₂ and 10% K₂CO₃, 1:1:2, by vol.). Normetanephrine and metanephrine stain a violet colour. They separate completely from all other substances as well as from each other.

When dry, the strips were scanned on a densitometer (Deniscord No. 552 Densitometer and


Diagnosis of phaeochromocytoma

Printer, slit aperture 1 mm × 6 mm, filter 505 nm, Photovolt Corporation). The area under each peak was automatically integrated and a digital value proportional to the amounts of separate normetanephrine and separate metanephrine was obtained. The standard strips were scanned in a similar fashion and a standard curve was plotted. The amounts of separate normetanephrine and separate metanephrine on the strips corresponding to 10 ml of urine used were obtained from the standard curve. The results were expressed as µg of separate normetanephrine (NM) or separate metanephrine (M)/mg of urinary creatinine.

Sensitivity, linearity and recovery studies
The smallest amount of NM that could be accurately estimated was 0.05 µg. The results are linear from 0.05 to 3.0 µg of NM, covering a convenient range. Determinations performed at different times usually require distinct base line and span (range) adjustments on the scanning densitometer.

Recovery studies were performed. Known amounts of NM were added to urine samples at different steps of the assay procedure. The assay procedures were then completed and the results compared with those obtained from the addition of known amounts of NM immediately before electrophoresis in which the recovery was 100%.

From these studies it was calculated that a loss of approx. 50% was incurred during the extraction procedure. There was no significant deviation from the mean value of recovery in spite of the large range of NM values studied.

Similar results were obtained when M was used instead of NM.

RESULTS
The results are summarized under two classifications, namely: hypertensive subjects and patients with phaeochromocytoma.

The chromatoelectrophoretic assay for combined metanephrines and 3-methoxy-4-hydroxy-phenylglycol was positive in all fourteen phaeochromocytoma cases (greater than 0.15 µg per 0.2 mg of creatinine). The spectrophotometric assay for combined metanephrines was also elevated (more than 1.5 µg per mg of creatinine). The two-dimensional chromatographic assay results for 3-methoxy-4-hydroxy-mandelic acid were increased in nine instances, indeterminate (borderline values were obtained) in three samples and normal findings were present in one specimen. The high-voltage electrophoretic assay results for separate normetanephrine and separate metanephrine were elevated (greater than 0.6 µg per mg of creatinine), in all of the cases except for two where a high value for only separate normetanephrine, indicating a norepinephrine secreting tumour, was obtained.

Of seventeen hypertensive patients who had false increased values for combined NM and M with the spectrophotometric assay, normal values were obtained for all other tests, including the chromatoelectrophoretic assay for combined NM and M and G, except in five instances when the two-dimensional chromatographic assay for VMA yielded one false high value and four false borderline values.

These results indicate that the most specific and accurate assay for establishing the diagnosis of phaeochromocytoma is the high-voltage electrophoretic assay for separate normetanephrine and separate metanephrine. The results for separate NM and separate M by employing this assay in the three groups of patients was always correct. The mean and standard deviation
values for NM for the normotensive, hypertensive and phaeochromocytoma groups, respectively, are: $0.079 \pm 0.034$, $0.163 \pm 0.123$ and $2.657 \pm 3.331$; the corresponding mean and standard deviation values for M, respectively, are: $0.105 \pm 0.067$, $0.180 \pm 0.108$ and $2.571 \pm 3.011$.

Despite the large range of values within each group of patients, the differences between the groups are highly significant ($P < 0.01$) and there is no overlapping of values between any of the groups.

**DISCUSSION**

The diagnosis of phaeochromocytoma is validly established by assay of the urine for the catecholamine metabolites, NM and M (Wolf et al., 1964, 1965, 1966; Wolf, Mendlowitz & Fruchter, 1970). It has clearly been shown that assay of the urine for 3-methoxy-4-hydroxy-mandelic acid to establish the diagnosis of phaeochromocytoma is comparatively inaccurate since false low and indeterminate results may be obtained in phaeochromocytoma patients and false elevated and indeterminate values have been demonstrated in non-phaeochromocytoma subjects both with and without associated hypertension. The incidence of false or indeterminate results with this test is approx. 15%.

The eventual criteria for the clinical usefulness of a diagnostic test for a specific disease must include the qualifications of unerring reliability and sensitivity together with simplicity of analysis and inviolate specificity. The high-voltage electrophoretic urinary assay for separate NM and separate M fulfills these standards. The simplicity of the test is defined by the fact that one laboratory technician using easily available laboratory apparatus can perform approx. thirty complete assays in two working days. The sensitivity of the test is demonstrated by the reliable detection of as little as 50 ng of either separate NM or separate M in a volume of 10-20 ml of urine. The specificity and reliability of the test are demonstrated by the precise, accurate results in sixty-seven urine specimens from patients with and without phaeochromocytoma. The inviolate accuracy of diagnosis with this new assay technique is compared with sixteen out of sixty-three false elevated results in non-phaeochromocytoma subjects with the spectrophotometric assay for combined NM and M and four false elevated results with the urinary assay for 3-methoxy-4-hydroxy-mandelic acid. Moreover, the urinary assay results for 3-methoxy-4-hydroxy-mandelic acid were clearly lower than normal in one case and indeterminate and disconcertingly confusing in four cases of phaeochromocytoma.

Happily, the urinary assay for combined NM and M employing chromatoelectrophoresis yielded the correct results in every specimen from patients with and without phaeochromocytoma.

It is recommended, therefore, that the diagnosis of phaeochromocytoma be entertained in every hypertensive patient. The diagnosis may be accurately established by assay of the urine for combined metanephrines employing the chromatoelectrophoretic test. Assay of the urine for separate NM and separate M to establish or refute the diagnosis of phaeochromocytoma may be employed and is recommended in any doubtful situation.

**ACKNOWLEDGMENTS**

Supported by Grants from the Health Research Council of the City of New York (V-1690) and from the Hypertension Research Fund.
REFERENCES


TANIGUCHI, K., KAKIMOTO, Y. & ARMSTRONG, M.D. (1964) Quantitative determination of metanephrine and normetanephrine in urine. *Journal of Laboratory and Clinical Medicine, 64*, 469–484.


