A Sensitive Double-Isotope Derivative Assay for Norepinephrine and Epinephrine

NORMAL RESTING HUMAN PLASMA LEVELS

By Karl Engelman, M.D., and Barry Portnoy, M.D.

With the Technical Assistance of Nancy Hurley, B.S.

ABSTRACT

A sensitive and specific double-isotope dilution method for assaying norepinephrine and epinephrine separately is described. The method is based on enzymatic conversion of these two catecholamines to their respective metanephrines and was applied successfully to measurement of resting plasma concentrations of norepinephrine and epinephrine in normal subjects with the finding that norepinephrine (0.20 ± 0.08 μg/liter) normally constitutes approximately 80% of the plasma catecholamine content and epinephrine (0.05 ± 0.03 μg/liter) 20%.

ADDITIONAL KEY WORDS

plasma catecholamines isoproterenol catechol-O-methyl-transferase alpha-methyl-norepinephrine

An enzymatic double-labeled isotope derivative assay for determining the absolute catecholamine content of biological specimens was previously developed in this laboratory (1). The method is sensitive and specific, and clinical application, especially to plasma samples, has proved feasible in a variety of conditions (2), but interpretation of results was limited by the inability to concomitantly measure norepinephrine and epinephrine independently in the same sample. This report will detail further modifications of the method which now enable differential assay of norepinephrine and epinephrine at nanogram levels, thus permitting the reliable determination of these compounds in 5 to 10 ml of human plasma, as well as in urine or other tissue samples.

Method

The assay technique is basically as described previously (1). The catecholamines are converted to their 3-O-methylated derivatives in the presence of tracer quantities of tritiated catecholamines, catechol-O-methyl-transferase (COMT) and S-adenosyl-L-methionine-methyl-14C (14C-AME). For separate assay of norepinephrine and epinephrine, the labeled metanephrines are then separated by thin-layer chromatography before scintillation counting.

Modifications of the published method include addition of both tracer 7-3H-DL-norepinephrine (3H-NE) and 7-3H-DL-epinephrine (3H-E) to the sample before processing, and the addition of 100 μg of both metanephrine and normetanephrine after incubation. Both plasma and urine catecholamines are isolated by chromatography on the IRC-50 columns originally described only for the plasma samples. The plasma samples are handled as originally described except that 1.0 ml 0.2M EDTA is added to the sample before it is placed on the column. A 0.5% aliquot of a 24-hour urine specimen is adjusted to pH 6.0-6.5 with NaOH after adding the labeled tracers, 1.0 ml 0.2M EDTA and 0.2 ml 0.6N ascorbic acid. The sample is then passed through the ion exchange column, followed sequentially by 50 ml of distilled water and 2 ml of 0.2N HCl. The catecholamines are then eluted into calibrated centrifuge tubes with 7 ml 0.2N HCl. The plasma eluates are lyophilized overnight while the urine eluate volumes are adjusted to 10 ml with distilled water. Urine eluates may be assayed immediately or stored at 4°C for a week.

The lyophilized plasma sample or 1.0 ml of the urine eluate is incubated in a mixture containing (final concn) ascorbic acid (10^-4M), MgCl₂ (5 x 10^-2M), 14C-AME (10^-5M), COMT (about 2 mg protein/ml) and phosphate buffer (5 x 10^-2M) made by adjusting 1.0M KH₂PO₄ to
pH 8.4 by adding freshly distilled N-ethyl morpholine.

To prepare the metanephrines for thin-layer chromatography, an IRC-50 column in the NH₄⁺ form rather than a Na⁺ form resin is used. After destruction of the unreacted tracer catecholamines by treatment with NaOH and heat, the incubation samples are adjusted to pH 6.0-6.5 by adding 1.0M acetic acid. The denatured proteins are sedimented by centrifugation (20,000 × g, 5 minutes) and the supernatant fluid is passed through an 11.5 × 40 mm column of Amberlite IRC-50, 100-200 mesh, NH₄⁺ form. (This resin is prepared by sequential cycling with 1.0N HCl, 1.0N NaOH and 1.0N HCl; after washing the resin free of excess acid, it is adjusted to pH 6.0 by mixing in 1.0M ammonium acetate, adding 4.0N NH₄OH as necessary to maintain a pH of 6.0). The columns are sequentially washed with HCl, followed by 1.0M acetic acid. The denatured proteins are sedimented by centrifugation (20,000 × g, 5 minutes) and the supernatant is applied in 100 ml distilled water followed by 0.05 ml absolute ethanol and mixed for 15 minutes (37°C). After centrifugation, the supernatant is applied in 7.0 ml 4.0N NH₄OH, 4.0N NaOH and 1.0N HCl; after washing the resin free of excess acid, it is adjusted to pH 6.0 by mixing in 1.0M ammonium acetate, adding 4.0N NH₄OH as necessary to maintain a pH of 6.0). The columns are sequentially washed with 100 ml distilled water and 3.0 ml 4.0N NH₄OH, and the metanephrines are then eluted in 7.0 ml 4.0N NH₄OH. After overnight lyophilization of the eluates the dried residue is suspended in 0.1 ml water followed by 0.05 ml absolute ethanol and mixed for 15 minutes (37°C). After centrifugation, the supernatant is applied in streaks (0.2 × 4.0 cm) on silica gel thin-layer chromatographic plates containing a fluorescence indicator (silica gel GF 250µ, Analtech Corp., Wilmington, Del.). The precipitate is again suspended in 0.05 ml 50% ethanol and the process is repeated; finally, the origin of each plate is coated with a streak of 20% NaCl. The chromatograms are developed for 2 hours in a solution of n-butanol: formic acid: water: 15: 2.5:: 1.5 (this system was suggested by Drs. John Daly and Robert Creveling). The plates are air dried and the two bands corresponding to metanephrine and normetanephrine are located by inspection under a short wave ultraviolet light in a darkened room. The dark bands containing the metanephrines are then scraped off the plates and mixed with 2.5 ml 4N NH₄OH for 30 minutes. After centrifugation, the supernatant material is removed and the silica gel is resuspended in a 2.0 ml water wash which is then removed and added to the NH₄OH eluate. The metanephrines contained in the supernatant are then converted to vanillin in a 45 ml extraction tube by incubation with 0.5 ml 3% NaNO₂ (25°C, 5 minutes). The sample is adjusted to pH 6.5 or less by adding glacial acetic acid, and the vanillin is extracted into 25 ml toluene by shaking for 15 minutes on a mechanical shaker. The tubes are centrifuged and the toluene layer is transferred to a 35-ml extraction tube containing 6.0 ml lm K₂CO₃. After shaking for 15 minutes, the K₂CO₃ layer is removed, acidified to less than pH 6.5 with glacial acetic acid and after evolution of CO₂ is completed, the vanillin is extracted into 14 ml toluene for scintillation counting. Computer programs have been written which permit rapid computation of the absolute catecholamine content in each sample. The specific activity of the ³¹C-AME and the "effective activity" of the ³¹H-NE and ³¹H-E must be determined for each different batch of labeled material used (1).

CLINICAL STUDIES

Two plasma samples were obtained on different days from supine, rested, fasting normal subjects (12 male, 10 female, age range 20-42 years). The subjects were laboratory personnel who rested for 20-30 minutes after arriving in the morning. This period of rest was sufficient to produce baseline values comparable to those of subjects whose blood samples were obtained in bed after awakening from sleep (2).

Results

Thin-layer chromatography permits rapid separation of metanephrine and normetanephrine; the final coating of the origin with NaCl increased separation and produced more compact bands. Under normal conditions the front migrates 14 to 16 cm during 2 hours and the Rf values (normetanephrine, 0.39; metanephrine, 0.27) result in excellent separation (Fig. 1). When 10 nmoles of norepinephrine and epinephrine were carried through the procedure there was less than 0.5% cross contamination from norepinephrine to epinephrine, and vice versa (Table 1). The radioactivity from an equimolar quantity of dopamine also contaminated the normetanephrine and metanephrine bands to the extent of only 0.7% and 0.3%, respectively. However, the β-hydroxylated catecholamine drugs, isoproterenol and α-methyl norepinephrine, both yield O-methylated metabolites which interfere significantly with the assay for norepinephrine but not epinephrine.

Data from more than 400 plasma assays indicate that the average assay recovery of tritiated tracer is 19% for the total catecholamine assay and 10 to 11% for the norepinephrine and epinephrine (Table 2). The sensitivity of the method is such that samples containing as little as 0.25 ng of norepinephrine or epinephrine can be measured accurately with a yield of ¹⁴C metanephrine counts, twice that of the assay blank. The average
values for plasma norepinephrine and epinephrine for each of the 22 subjects are plotted in Figure 2. The group value (mean ± SD) for plasma norepinephrine was

| TABLE 1 |

| Thin-Layer Chromatography for the Separation of O-methylated Catecholamines |
|---------------------------------|-------------------------------|-------------------------------|
| Substrate                        | 14C-dpm/10 nmoles substrate   |                           |
|                                 | Normetanephrine band         | Metanephrine band          |
| L-norepinephrine                 | 245,989                       | 1,733                       |
| L-epinephrine                    | 1,138                         | 189,248                     |
| Dopamine                         | 1,738                         | 478                         |
| Dl-isoproterenol                 | 252,345                       | 2,178                       |
| L-a-methyl norepinephrine        | 147,515                       | 3,117                       |

| TABLE 2 |

| Sensitivity of Isotope Assay for Plasma Catecholamines |
|---------------------------------|-------------------------------|-------------------------------|
| Assay                           | % Recovery (Mean, range)     | Average yield above blank (14C-dpm/ng) | Average blank (14C-dpm) |
| Total catecholamines            | 19 (7-23)                     | 78                           | 34                      |
| Norepinephrine                  | 10 (3-20)                     | 42                           | 11                      |
| Epinephrine                     | 11 (4-20)                     | 44                           | 11                      |
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0.20 ± 0.08 μg/liter (range 0.10-0.37) and that for plasma epinephrine was 0.05 ± 0.03 μg/liter (range 0.00-0.11). The data for norepinephrine and epinephrine from the 10 females and 12 males were compared (Fig. 2) and there was no statistically significant difference for each (norepinephrine \( P > 0.6 \), epinephrine \( P > 0.1 \)) Total plasma catecholamines (norepinephrine plus epinephrine) assayed on duplicate samples from the same individuals (2) yielded values \( 0.24 ± 0.06 \) μg/liter), which were in excellent agreement with the sum of the values for norepinephrine and epinephrine measured separately.

Discussion

The double-isotope enzymatic derivative method as originally described for assay of total catecholamines (1) introduced a new order of specificity and sensitivity to catecholamine assays while also providing the first method of catecholamine analysis to permit a true recovery to be determined on each sample. In other methods a recovery may be run on a duplicate sample, but in no other procedure is it possible to determine the recovery on the same sample. The modifications described in this paper now extend this method to the independent assay of norepinephrine and epinephrine and this method now permits more accurate differential determination of these catecholamines than has been available heretofore. Whereas the standard spectrophotofluorimetric assays for norepinephrine and epinephrine are based on the difference in the relative fluorescent yields of these two catecholamines after oxidation at pH 6.5 and 3.5 (4, 5) or on minor differences in the overlapping fluorescent spectra of their trihydroxyindole derivatives (6), this isotopic assay is based on the actual physical separation of their O-methylated derivatives, normetanephrine and metanephrine. Even under optimal conditions the differential determination of norepinephrine and epinephrine in the fluorimetric assays is inaccurate for the amine whose content is less than 10% of the other. In contrast, in this assay the physical separation of normetanephrine and metanephrine by thin-layer chromatography allows cross contamination of these two amines to the extent of only 0.5% or less, thus permitting reliable assay of mixtures of norepinephrine and epinephrine over a very wide range of relative concentrations.

The high degree of specificity of the method was originally shown for a large variety of catechol and related phenolic compounds (1). This specificity derives from the number of separate, selective steps employing ion-exchange resin, solvent-partition and thin-layer chromatography as well as the specific enzyme reaction with COMT and the periodate cleavage reaction, which depends on the presence of the \( \beta \)-hydroxyl group. Despite these techniques it is evident that the drug isoproterenol or alpha-methyl-norepinephrine, a metabolite of the drugs alpha-methyl dopa (7) and alpha-methyl-tyrosine (8), will still present difficulties in assay of specimens from humans or animals receiving these drugs. However, as we originally proposed, appropriate modification of these techniques should permit the assay of any catechol compound. A recent preliminary communication has already reported a modification enabling the assay of dihydroxymandelic acid in human urine (9).

Although we have determined the content of these amines in samples of 5-10 ml plasma obtained in the resting state from normal subjects, epinephrine levels still cannot be measured very accurately at the lower range of normal. Any increase in plasma catecholamine concentration makes assay easier, and the technique has already been applied successfully to the measurement of total plasma catecholamines in preliminary studies of exercise, mental depression, acute myocardial infarction and essential hypertension (2, and unpublished observations), indicating the feasibility of such studies at both the animal and human level. Furthermore, we have found the method to be applicable to studies of urinary catecholamine excretion and to the assay of tissue levels as well. Though the sensitivity offered by this assay is not ordinarily required for urine samples or for assay of

Circulation Research, Vol. XXVI, January 1970
tissues with high catecholamine concentrations, the unique built-in recovery factor recommends this method as a new standard technique.

References
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Circ Res. 1970;26:53-57
doi: 10.1161/01.RES.26.1.53
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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