An Automated Trihydroxyindole Procedure for the Differential Analysis of Catecholamines

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An automated trihydroxyindole procedure for the differential estimation of epinephrine and norepinephrine in tissues, urine, and blood is presented in detail. After an initial purification of the catecholamines by adsorption on aluminum oxide, the neutralized eluates are subjected to automatic analysis, in which potassium ferricyanide and an alkaline stabilizing agent are automatically mixed sequentially with the dialyzed catecholamines. The fluorescent trihydroxyindole derivatives formed are then delivered to a flow cell in a fluorometer and the fluorescence recorded. A differential estimation is obtained by running samples through the system, first with ascorbic acid stabilization to obtain the combined fluorescence of both amines and then with thioglycolic acid stabilization to obtain the fluorescence of norepinephrine alone.

Fluorescent trihydroxyindole derivatives are formed when the catecholamines, in a slightly acid solution, are oxidized by potassium ferricyanide or iodine and then subjected to a strongly alkaline solution containing an antioxidant. The antioxidant is necessary to stabilize the fluorescent derivatives. Various modifications of this procedure are widely used for the estimation of epinephrine and norepinephrine in extracts of tissues, urine, and blood (1-3). A differential estimation of epinephrine and norepinephrine in mixtures can be achieved by taking advantage of differences in the rates of their reaction when oxidation is carried out at two pH's (2-5) or in the differences in their excitation-emission characteristics when two sets of filters are used (1, 6).

Merrills (7, 8) has adapted the trihydroxyindole method for automatic analysis using an AutoAnalyzer* and a modified Locarte fluorometer.† Purification of the catecholamines by adsorption on aluminum oxide, elution with acetic acid, and neutralization of the eluates are carried out

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by conventional manual procedures. The use of the differential pH or filter method for the estimation of epinephrine and norepinephrine in mixtures is not feasible with the automated procedure. However, Merrills (7, 8) has made the important discovery that when thioglycolic acid is substituted for ascorbic acid in the alkali-antioxidant mixture, only the fluorescent product of norepinephrine is stabilized. An aliquot of a sample is run with ascorbic acid as the stabilizing agent to obtain the combined fluorescence of epinephrine and norepinephrine, and the run is repeated with thioglycolic acid to obtain the fluorescence of norepinephrine only. The epinephrine is then calculated by difference.

We have adapted this method to the AutoAnalyzer commercially available in this country with only slight modification of Merrills' original method. The present paper describes this modification and its application to the analysis of catecholamines in several tissues, urine, and blood.

**Methods**

**Apparatus**

The AutoAnalyzer is used with a Turner fluorometer adapted by the manufacturer of the AutoAnalyzer for use with their equipment. A Turner blue lamp (No. 110-853*) is used as the source of ultraviolet light. The primary filter used in the fluorometer is the Turner No. 47-B narrow pass filter,* which peaks at 436 m$. The secondary filter is the Turner No. 2A-12 sharp cut filter,* which passes all wave lengths longer than 510 m$.

**Reagents**

1. **Aluminum oxide** (Woelm Neutral Activity Grade 1 or British Drug Houses Ltd. "for chromatographic adsorption analysis")† Prepare the former by the method described by Crout (2), and use the latter without further treatment.

2. **Sodium acetate buffer, 0.1 M, pH 6** Sodium acetate (0.1 M) is adjusted to pH 6 with acetic acid.

3. **Sodium carbonate-bicarbonate buffer** Dissolve 80 gm. NaHCO$_3$ and 80 gm. Na$_2$CO$_3$ in water and dilute to 1 L.

4. **Phosphate buffer, pH 12** Add 60 ml. 3 N NaOH to 100 ml. 1 M NaH$_2$PO$_4$.

5. **Acetate Buffer, 0.05 M, pH 6** Adjust 0.05 M sodium acetate to pH 6 with acetic acid.

6. **Potassium Ferricyanide, 0.005%** Dissolve 50 mg. K$_3$Fe (CN)$_6$ in 0.05 M acetate buffer, pH 6, diluting to 1 L.

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†Available from The Ealing Corp., Cambridge, Mass.
7. Sodium hydroxide, 2.5 N  Dissolve 100 gm. NaOH in water and dilute to 1 L.
8. Ascorbic acid, 0.15%  Dissolve 150 mg. ascorbic acid in water, diluting to 100 ml.
9. Thioglycolic acid (Mercaptoacetic acid, Eastman), 1% v/v  Dissolve 1 ml. thioglycolic acid in 100 ml. water.
10. Disodium ethylenediaminetetraacetate (EDTA)
11. Sodium metabisulfite
12. Thymol blue WS (Nutritional Biochemicals Corporation)
13. Octanoic acid (Eastman)
14. Epinephrine stock standard, 1000 µg. base per milliliter  Dissolve 18.2 mg. l-epinephrine bitartrate (Winthrop) in 10.0 ml. 0.01 N hydrochloric acid.
15. Norepinephrine stock standard, 1000 µg. base per milliliter  Dissolve 19.9 mg. l-norepinephrine bitartrate monohydrate (Winthrop) in 10.0 ml. 0.01 N hydrochloric acid.
16. Working standards, 0.1 µg./ml.  Dilute 1 ml. of each stock standard to 100 ml. with water. Dilute 1 ml. of the first dilution of each standard to 100 ml. with 0.1 M acetate buffer, pH 6.
17. Water  The water used in all steps is distilled water which has been passed through a commercial deionizer.

All chemicals used were of the highest grade obtainable and, with the exception of the Woeim aluminum oxide, further purification was not necessary.

Preparation of Samples

Tissues

Excise, weigh, and homogenize tissues in 10 volumes of 0.4 N perchloric acid in a glass Tenbroek tissue grinder submerged in an ice bath. Centrifuge the homogenates at 1000g for 15 min. at 4°, and add 10 mg. sodium metabisulfite to the supernatants. The catecholamines in the supernatants are stable for at least 1 week if frozen.

Urine

To a 2-hr. or other suitable urine specimen add sodium metabisulfite (0.5 mg./ml.), filter, and adjust the pH to 3.5 with 10% (v/v) sulphuric acid. Store a 40-ml. aliquot at 4°. Filter the 24-hr. output of rat urine, adjust to pH 3.5, dilute to 40 ml. with water, and store at 4°.

Blood

Collect up to 40 ml. of heparinized blood in chilled tubes containing sodium metabisulfite (0.5 mg./ml.) and immediately centrifuge at 1000g
for 25 min. Precipitate the proteins by adding 0.1 ml. of cold 4 N perchloric acid per milliliter of plasma and mixing vigorously. Place the tubes in a refrigerator at 4° for 1/2 hr. and then centrifuge at 1000g for 15 min. Collect and freeze the supernatants.

**Adsorption of Aluminum Oxide**

To each cold extract add 200 mg. EDTA and 2 drops of thymol blue indicator. Adjust the pH to 8.3–8.5 by the dropwise addition of sodium carbonate-bicarbonate buffer from a buret until a very faint blue color appears. Adjust the pH of urine specimens with a pH meter. Keep the solution in continual vigorous agitation during this time, using a magnetic stirrer. Prepare the column by suspending 1 gm. of aluminum oxide in water and pouring it into a glass column of 1-cm. bore with a sintered glass filter near its lower end. Allow the aluminum oxide to settle and remove air bubbles by stirring gently with a glass rod. Wash the column with 20 ml. of water. Carefully add the extract at pH 8.3–8.5 to the column and pass through the aluminum oxide at a rate of about 1 ml./min. Apply air pressure from above if necessary to obtain this rate of flow. After the extract has passed through the aluminum oxide, wash the column twice with 20 ml. of water at the same rate.

If unwashed British Drug Houses Ltd. aluminum oxide is used, place 1 gm. in a 50-ml. beaker and suspend in water 4 or 5 times, decanting each wash to remove small particles. Then pour the aluminum oxide into the glass column, add the extract, and allow to pass through by gravity. After the extract has passed through the aluminum oxide, wash the column 10 times with 20 ml. of water. The rate of flow of the washes may be accelerated slightly by applying air pressure from above. The larger number of washes does not appreciably lengthen the preparation time, since a number of columns can be set up and handled almost simultaneously. Because British Drug Houses aluminum oxide is more convenient to use and gives more consistent recoveries we recommend its use.

**Elution**

Add 4 ml. of 0.2 N acetic acid to the column and briefly stir the aluminum oxide with a glass rod. Allow the acid to pass through the column by gravity and follow with 4 ml. of water. The collected eluate is frozen until just before the assay is carried out.

**Analysis with the AutoAnalyzer**

Before placing the eluates in the AutoAnalyzer sampler cups, adjust them to pH 6 with phosphate buffer, using a pH meter. Then centrifuge at 1000g for 10 min. to remove the precipitate of aluminum phosphate.
which appears. If the eluates must be diluted to bring their catechol-
amine concentrations within the range of the standards (0.1 \(\mu g./ml.\)) use 0.1 M acetate buffer, pH 6. Determine the volumes of the eluates and fill the 2-ml. sampler cups to a constant level.

The flow diagram of the AutoAnalyzer used in the fluorometric deter-
mination of epinephrine and norepinephrine is shown in Fig. 1. The

![Flow diagram of automatic system](image)

**Fig. 1.** Flow diagram of automatic system, showing tube sizes and concentrations of reagents. Water and potassium ferricyanide are saturated with octanoic acid. Double dialysis is employed. This is accomplished by connecting two sets of dialysis plates, with C-type membranes, in series.

The manifold was developed in conjunction with the manufacturer of the AutoAnalyzer and can be obtained from them. In practice, two sets of dialysis plates with Type C membranes are connected in series. The samples and water (saturated with octanoic acid) are pumped separately, mixed, and delivered to the upper surface of the dialysis membrane. Buffered potassium ferricyanide (saturated with octanoic acid) is pumped to the lower surface of the membrane. The catecholamines which pass through the membrane into the recipient stream (25%) are oxidized and emerge from the dialyzer as the corresponding chromogens, while all nondialyzable materials in the samples are excluded. Since the
alkali and stabilizing agent constitute a relatively unstable solution, they
are picked up separately and mixed in the AutoAnalyzer just before
reaching the stream containing the oxidized catecholamines. The trans-
formation of chromogens into the corresponding fluorescent lutines takes
place in the double mixing coil. The lutines are then delivered to the flow
cell in the fluorometer where all air is removed before the sample reaches
the light path. The resulting fluorescence is recorded.

**Differentiation of Epinephrine and Norepinephrine**

In order to estimate differentially the epinephrine and norepinephrine
contents of samples, pass the samples through the AutoAnalyzer, first
with ascorbic acid, and then with thioglycolic acid as the stabilizing
agent. The fluorescence recorded during the first run is due to the com-
bined fluorescence of epinephrine and norepinephrine, while the fluo-
rescence recorded during the second run is due only to norepinephrine. The
epinephrine and norepinephrine per milliliter of the sample aliquots
tested can be calculated using appropriate formulas (see below, under
*Calculations*). Samples adjusted to pH 6 have been found to be stable
for several hours at room temperature.

When ascorbic acid is used as the stabilizing agent set the sensitivity
of the fluorometer at full scale and use Slit 3. When the stabilizing agent
is thioglycolic acid set the sensitivity at full scale with maximum slit
width.

The AutoAnalyzer is run at 40 samples per hour with a cup containing
water placed between each sample cup. Thus up to 20 samples per hour
can be run. Epinephrine and norepinephrine standards containing 0.1
μg. of the amine per milliliter are run at frequent intervals to establish
the calibration of the fluorescence and to check for changes in sensitivity.
A slow decrease in sensitivity amounting to 1–2% per hour has been
noted.

There is no detectable contamination from sample to sample going
from high to low values of fluorescent intensity when cups containing
water are placed between samples.

**Calculations**

Norepinephrine is determined directly from the fluorescence reading
obtained on the aliquot of the diluted eluate run with thioglycolic acid
as stabilizing agent. The following formula is used:

\[ \frac{0.1 \cdot F_r}{S_r} = \mu g. \text{ norepinephrine/ml.} \]

where \( F_r \) is the fluorometric reading of sample aliquot run with thiogly-
colic acid, corrected for reagent baseline, and \( S_r \), the fluorometric reading
of standard norepinephrine run with thioglycolic acid, corrected for reagent baseline.

The amount of epinephrine in the aliquot is calculated with the following formula:

\[ \frac{B_a \cdot NE \cdot Y_a}{X_a} = \mu g. \text{ epinephrine/ml.} \]

where \( B_a \) is the fluorometric reading of sample aliquot run with ascorbic acid, corrected for reagent baseline, \( NE \), norepinephrine in micrograms per milliliter; \( Y_a \), fluorometric reading of norepinephrine standard run with ascorbic acid, corrected for reagent baseline, divided by concentration of the norepinephrine standard (0.1 \( \mu g./ml. \) ); and \( X_a \), fluorometric reading of epinephrine standard run with ascorbic acid, corrected for reagent baseline, divided by concentration standard (0.1 \( \mu g./ml. \) ).

The amounts of epinephrine and norepinephrine in the eluates from aluminum oxide are obtained by multiplying the values calculated above by the extent (milliliters) to which the eluates were diluted.

**Calibration**

As shown in Fig. 2 and 3 the calibration curve for both epinephrine and norepinephrine is linear. When ascorbic acid is the stabilizing agent

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**Fig. 2.** Example of record obtained when different concentrations of epinephrine and norepinephrine standards are run through system during ascorbic acid stabilization. Concentrations are in micrograms per milliliter.
the fluorescence of the norepinephrine standard is approximately 40% of that of epinephrine standard. When thioglycolic acid is the stabilizing agent the fluorescence of the epinephrine standard is less than 1% of that of the norepinephrine standard.

Blank

A faded sample blank can be obtained by replacing the ascorbic acid or thioglycolic acid with water and rerunning all samples. A blank may also be obtained by replacing potassium ferricyanide with a 0.1% thioglycolic acid solution. The thioglycolic acid is necessary because some oxidation of catecholamines occurs within the AutoAnalyzer system even in the absence of potassium ferricyanide. If this type of blank is utilized it should be the last operation of the day, since water must be run through the entire system for several hours to remove all traces of thioglycolic acid.

Recovery

Recovery of epinephrine and norepinephrine from acid is 95–100%. When the catecholamines in acid are passed through acid-washed Woelm aluminum oxide, recovery is between 75 and 90%. Recovery of the catecholamines from extracts of tissues, urine, and blood is 70–90%. No consistent difference in the recovery of epinephrine and norepinephrine

Fig. 3. Standard curves of epinephrine (E) and norepinephrine (NE), with ascorbic acid and thioglycolic acid stabilization.
is noted. When unwashed British Drug Houses aluminum oxide is used, recoveries are more consistent, ranging between 71 and 80%.

**Results**

The record obtained in the analysis of the catecholamine contents of three rat hearts is shown in Fig. 4. Epinephrine in these three hearts ranged between 6.0 and 12.0% of the total catecholamine content.

Figure 5 is a record showing the effect on the rate of epinephrine release of adding tyramine to the fluid perfusing an isolated dog adrenal. The effluent was tested directly without adsorption on aluminum oxide. The standard epinephrine was made up in the perfusion medium and the analysis carried out with ascorbic acid stabilization. It has previously been shown that tyramine does not change the ratio of epinephrine to norepinephrine released from the isolated adrenal (9). Tyramine does not fluoresce and does not interfere with the analysis.

The results of the analysis of the catecholamine contents of several tissues and urine are shown in Table 1. The results obtained are in good agreement with those reported in the literature (3, 10, 11, 12).

In general, catecholamines could not be estimated in peripheral arterial blood samples taken from dogs under pentobarbital anesthesia.
However, catecholamine concentrations could be measured in animals subjected to stress. For example, in a dog whose mean blood pressure had been lowered to 40 mm. Hg by hemorrhage, the peripheral blood catecholamine concentration rose from an undetectable value to 7.5 μg./L. and 0.2 μg./L. for epinephrine and norepinephrine, respectively.

We have consistently found that tissue and plasma samples which have been adsorbed on aluminum oxide give no blank reading. Occasionally small blanks were observed with urine samples, but these were never more than 5% of the sample reading.

**Discussion**

We have been able to reproduce satisfactorily Merrills’ (7, 8) automated trihydroxyindole procedure for the estimation of epinephrine and

![Fig. 5. Tyramine release of catecholamines from isolated dog adrenal.](image)

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<thead>
<tr>
<th>Table 1. Estimated Epinephrine and Norepinephrine in Urine and Tissues</th>
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<tbody>
<tr>
<td><strong>No. of animals</strong></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Human urine (μg./24 hr.)</td>
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<tr>
<td>Rat urine (μg./kg./24 hr.)</td>
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<tr>
<td>Rat heart (μg./gm.)</td>
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<tr>
<td>Rat submaxillary gland (μg./gm.)</td>
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<td>Rat adrenal gland (μg./100 mg.)</td>
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*Means ± standard errors of the means.*
norepinephrine using commercially available equipment. Although 0.3% ascorbic acid as recommended by Merrills is satisfactory, we have chosen to use 0.15% ascorbic acid because the reagent baseline is slightly reduced without any apparent decrease in sensitivity or stability of the fluorescent products.

It is unfortunate that no one has been able to automate the procedure for the adsorption and elution of catecholamines on aluminum oxide columns. Despite this, the automation of the analytical procedure has many obvious advantages over the manual methods. Many investigators (2, 3, 8, 13) have pointed out the disadvantages of the manual methods. Accurate timing of the addition of reagents and of the reading of the fluorescence is important and difficult to reproduce manually. This problem is completely eliminated when the automated system is employed. The instability of ascorbate in a strongly alkaline solution results in a progressive increase in the fluorescence of the reagent background and sample. In the automated procedure ascorbic acid and NaOH are picked up separately and mixed in the system just prior to being mixed with the oxidized chromogens, thus minimizing this problem. Another problem that frequently arises with the manual methods is the occurrence of turbidity in the final solution which can lead to light-scatter and quenching of fluorescence. Dialysis of samples eliminate dust and any particulate matter, as well as large molecules, from the final solution and consequently greatly reduces turbidity and quenching. The efficacy of dialysis is demonstrated by the fact that we have confirmed Merrills' observation that a "faded blank" is not required with tissue or plasma samples which have been purified on aluminum oxide columns.

The ability of thioglycolic acid to stabilize selectively the fluorescence of norepinephrine makes the automated method uniquely qualified for the differential estimation of epinephrine and norepinephrine. In all other fluorometric methods it is the fluorescence of norepinephrine which is suppressed while that of epinephrine is maintained. In general the ability of fluorometric methods to differentiate between epinephrine and norepinephrine is poor because the relative fluorescence of the amines under the two sets of conditions used for differentiation is not very different. The automated method gives widely divergent ratios of fluorescence for the two amines with the two different stabilizing agents and is thus capable of resolving them more completely than other fluorometric methods. Only the method described by Crout (2), which depends on iodine oxidation at two different pH values, rivals the automated method for differential measurement of epinephrine and norepinephrine.
The automated trihydroxyindole procedure is suitable for the routine clinical diagnosis of pheochromocytoma and is a valuable research tool for the quantitative measurement of epinephrine and norepinephrine.

References