The Determination of Catechol Amines in Blood

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The growing clinical interest in the action and metabolism of the catechol amines makes it highly desirable to re-evaluate existing methods for the quantitative determination of these substances in body fluids. In 1955 Persky (1) reviewed the subject, and, on the whole, his conclusion is still valid that the fluorometric determination of the catechol amines in body fluids is the most practical method for clinical purposes. For the determination of the two catechol amines in the presence of each other, the methods of Lunt (2, 3) and of Weil-Malherbe (4), and modifications thereof, are used most frequently. The former depends upon the differential oxidation of the catechol amines at pH 3.5 and 6.5 and the anaerobic rearrangements of the resulting o-quinones to the intensively fluorescing "lutin" derivatives. In the Weil-Malherbe method the fluorescence is stabilized by coupling the quinones in the status nascendi with ethylene diamine, and measuring the emitted fluorescence at two different wave lengths. According to Persky (1), methods depending upon the ethylene diamine condensation are somewhat more reliable than those involving oxidation at different pH values. However, the precision of this method depends to a large extent on the assumed constancy of the fluorescence in the region between 510 and 600 mμ (5).

Recently Goldfien and Karler (6) demonstrated the effect of light on the fading of the fluorescence of the ethylene diamine condensation products of the oxidized catechol amines. Studies carried out in this laboratory during the last year fully confirm these results.
and form the basis of a method for the determination of epinephrine and norepinephrine in blood to be reported here.

**REAGENTS AND METHOD**

**REAGENTS**

1. Distilled water. All solutions must be made up in glass-distilled water and must be free of nonspecific fluorescence.
2. Acetic acid (0.1M)
3. Acetic acid (0.2M)
4. Sodium acetate (0.2M, pH 8.4). Adjust the pH with 0.5M sodium carbonate.
5. Alumina, acid washed. Stir 100 Gm. of alumina (Adsorption Grade, Fisher Scientific Co.) with 500 ml. of boiling 2N hydrochloric acid and wash on a sintered glass funnel with 500 ml. of hot hydrochloric acid. Wash repeatedly with distilled water until the supernatant is no longer acid. Activate at 300° for three hours.
6. Ethylene diamine dihydrochloride (2M). To about 50 ml. of double distilled water add 16.6 ml. concentrated hydrochloric acid and 13.3 ml. ethylene diamine (caution: fuming will occur). Dilute to 100 ml. Make up fresh daily.
7. Ethylene diamine. Redistill immediately prior to use or redistill and store in the frozen state.
8. n-Butyl alcohol. Redistill prior to use or store in a deep freeze after redistillation.
10. Stock epinephrine standard (0.5 mg./ml.). Dissolve 91 mg. epinephrine bitartrate in 100 ml. of 0.01N hydrochloric acid and store in a refrigerator.
11. Stock norepinephrine standard (0.5 mg./per ml.). Dissolve 99.5 mg. norepinephrine bitartrate in 100 ml. of 0.01N hydrochloric acid and store in a refrigerator.
12. Working epinephrine standard (0.1 µg. per ml.). Made up fresh daily in 0.1N acetic acid from stock standard.
13. Working norepinephrine standard (0.1 µg/ml.). Prepared fresh daily in 0.1N acetic acid from the stock standard.

**EQUIPMENT**

Spectrofluorometer, Farrand. In order to obtain greater stability a Keithly Model 410 micro-microammeter was substituted for the RCA ultra-sensitive microammeter normally supplied with this spec-
trofluorometer. The 1P28 photomultiplier tube was replaced by a 1P21 photomultiplier tube for the same reason. A tilting platform type (VirTis) automatic extractor was used for the butanol extraction of the ethylene diamine derivatives. A column 6 mm. x 150 mm. with a reservoir 51 mm. x 120 mm. on top was used for the adsorption of the catechol amines on alumina. Screw cap culture tubes 16 mm. x 150 mm. were used to collect the eluate. The caps of these tubes were lined with polythene plastic.

PROCEDURE

The alumina column is prepared from a slurry of 1 Gm. of activated alumina in 10 ml. of distilled water. Throughout the entire procedure the level of the liquid should never be lower than one-fourth inch above the alumina. After 5 ml. of sodium acetate buffer has drained to this level the column is ready for the sample. For best results 10 ml. of heparinized plasma should be used. The plasma is mixed with an equal volume of sodium acetate buffer and run through the column at the rate of one drop every two or three seconds. The column is then washed with 5 ml. acetate buffer followed by two 5 ml. portions of double distilled water. The catechol amines are eluted with 5 ml. of 0.2M acetic acid followed by 5 ml. double-distilled water.

At this point the room is partially darkened by turning the room lights off and closing the blinds. If needed, the tubes are covered to prevent the fading of the fluorescence of the condensation products which occurs in the presence of light. For the coupling reaction 0.7 ml. of ethylene diamine and 0.5 ml. ethylene diamine dihydrochloride are added to the 10 ml. eluate. The tubes are placed in a darkened water bath at 50° for 20 minutes and then transferred to a beaker of cold tap water for five minutes. After saturation with sodium chloride (5-7 Gm.), 4 ml. of butanol is added. The tubes are shaken for ten minutes on an automatic extractor. The emitted fluorescence of the butanol layer is measured at 510 m\(\mu\), using 436 m\(\mu\) as the exciting wave length. From this point on, all work may be carried out in the presence of light. Now the tubes are stoppered and placed near a "daylight" bulb such as is found in an x-ray film viewing box. After 30 minutes exposure to this light source, the residual fluorescence is measured at 510 m\(\mu\). A reagent blank and known amounts of each of the catechol amines are carried through all the steps along with the plasma sample.
CALCULATIONS

The amounts of epinephrine and norepinephrine in the plasma samples are calculated from the fluorometric readings of the sample and of known amounts of epinephrine and norepinephrine before and after exposure to light. After subtracting the readings for the reagent blank the corrected readings are substituted in the following equations:

\[ R_s + R_n = R_1 \]  \hspace{1cm} (1)
\[ xR_s + yR_n = R_2 \]  \hspace{1cm} (2)

Where:
\( R_s \) = Fluorescence due to epinephrine in sample.
\( R_n \) = Fluorescence due to norepinephrine in sample.
\( R_1 \) = First reading of sample in fluorometer.
\( R_2 \) = Second reading of sample in fluorometer.
\[ x = \frac{\text{Second reading of epinephrine standard}}{\text{First reading of epinephrine standard}} \]
\[ y = \frac{\text{Second reading of norepinephrine standard}}{\text{First reading of norepinephrine standard}} \]

Solution of simultaneous equations (1) and (2) gives the values for \( R_s \) and \( R_n \) as shown in equations 2A and 2B.

\[ (2A) \quad R_s = \frac{R_2 - yR_1}{x - y} \]
\[ (2B) \quad R_n = \frac{xR_1 - R_2}{x - y} \]

\[ E = \frac{R_s \times \mu g \text{ epinephrine in standard}}{\text{Initial reading of epinephrine standard}} \]  \hspace{1cm} (3)
\[ N = \frac{R_n \times \mu g \text{ norepinephrine in standard}}{\text{Initial reading of norepinephrine standard}} \]  \hspace{1cm} (4)

Where:
\( E \) = Amount in \( \mu g \) of epinephrine in plasma sample used.
\( N \) = Amount of norepinephrine in \( \mu g \) in plasma sample used.

Substitution of the values for \( R_s \) and \( R_n \) in equations (3) and (4) will give the amounts of catechol amines in the sample.
DISCUSSION OF RESULTS

The determination of the catechol amines involves the quantitative separation and estimation of two closely related substances in the presence of each other and a multitude of interfering substances. Since the catechol amines are present in normal individuals in about $2 \times 10^{-11}$ molar concentrations, it is obvious that any method for their quantitative determinations is fraught with many technical difficulties. Chief among these is the nonspecific fluorescence picked up by the water, glassware, or butyl alcohol. During the course of this study all reagents and solvents were routinely tested for such fluorescence, and if present it was removed or reduced by distillation or recrystallization of the reagents until they showed a minimum fluorescence. All glassware, obviously, has to be kept scrupulously clean.

In the Weil-Malherbe method and its modification (1) no particular attention is paid to the exposure of the ethylene diamine complex to daylight during the determination. However, the results obtained in this laboratory as shown in Fig. 1 fully confirm the report of Goldfien and Karler (6) that the ethylene diamine complex of the o-quinone derivative of norepinephrine is strongly affected by exposure to light. In the present study, samples of epinephrine and norepinephrine (0.1 µg per ml.) were carried through the steps outlined under PROCEDURE. It will be noticed that the fluorescence of the ethylene diamine complex of the quinone derivative of norepinephrine before exposure to light showed a maximum at 485 mµ with the exciting light at 436 mµ. On the other hand, after exposure to a daylight bulb for 30 minutes nearly all fluorescence disappears. This latter curve approaches the values reported by other investigators (6) for the ethylene diamine complex of the quinone derivative of norepinephrine. In the case of the epinephrine derivative it can be seen that with an exciting light beam at 436 mµ the emitted fluor-

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**Fig. 1.** Fluorescent spectra of epinephrine and norepinephrine with exciting light beam at 436 mµ.

1. Epinephrine before exposure to light.
2. Epinephrine after exposure to light for 30 minutes.
4. Norepinephrine after exposure to light for 30 minutes.
escence peaks at a 530 m. It will also be noticed that the fluorescence of this compound fades only about 15% as the result of exposure to light for 30 minutes. To correct for the slight fading of the epinephrine compound and the slight residual fluorescence of the norepinephrine derivative, known amounts of the two catechol amines are carried through the procedure along with the plasma sample. There is, therefore, a difference in principle between previous methods and the proposed procedure. In the former the catechol amines are quantitated by measuring the residual fluorescence of the o-quinone-ethylene diamine complexes at two different wave lengths after an uncontrolled exposure to light. The basis of the proposed method, on the other hand, is the difference in the stability of the fluorescence at a single wave length shown by the two complexes under conditions of controlled exposure to light.

Table 1 shows some of the results of recovery studies carried out with the proposed method. Known amounts of each of the catechol amines were added to samples of pooled serum. The original serum and the fortified samples were then analyzed for each of the catechol amines. The results as shown in the table indicate that the recovery is about ± 20 per cent of the expected value.

In this connection it should be pointed out that both epinephrine and norepinephrine are easily destroyed in shed blood. This is well demonstrated in Table 2. In this experiment known amounts of both

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Serum</th>
<th>Catechol Amines Added*</th>
<th>Catechol Amines Present*</th>
<th>Catechol Amines Found*</th>
<th>Percentage Recovery</th>
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<tbody>
<tr>
<td>I</td>
<td>N*</td>
<td>.04</td>
<td>.20</td>
<td>.24</td>
<td>.23</td>
</tr>
<tr>
<td></td>
<td>E*</td>
<td>.00</td>
<td>.20</td>
<td>.20</td>
<td>.21</td>
</tr>
<tr>
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<td>N</td>
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<td>.20</td>
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<td>.20</td>
<td>.20</td>
<td>.20</td>
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</tbody>
</table>

N* = Norepinephrine
E* = Epinephrine
* = All values expressed in μg per 5 ml. of serum.
of the catechol amines were added to serum samples. One aliquot of each sample was then analyzed immediately by the proposed method. Another aliquot was allowed to stand at room temperature for three hours and then was analyzed for each of the catechol amines. A third aliquot was kept in a deep freeze for 24 hours, allowed to thaw at room temperature and carried through the procedure. It will be seen that when analyzed immediately, a recovery of better than 80% was obtained. On the other hand, when kept at room temperature for three hours nearly all of the catechol amines were destroyed. The frozen aliquot lost about 60 per cent of its activity, presumably during the process of thawing.

The catechol amine levels found in normal individuals consisting of laboratory personnel and medical students are shown in Table 3. From these results it appears that the normal level of norepinephrine by the proposed method is from 0.3-4.0 µg per liter of plasma, and the epinephrine values range from 0.1-1.5 µg per liter.

Attempts to apply this method to the determination of urinary
Table 3. Catechol Amine Values of Normal Individuals

<table>
<thead>
<tr>
<th></th>
<th>Norepinephrine µg/liter of plasma</th>
<th>Epinephrine µg/liter of plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.A.H.</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>B.A.R.</td>
<td>1.7</td>
<td>0.3</td>
</tr>
<tr>
<td>B.O.B.</td>
<td>0.7</td>
<td>1.3</td>
</tr>
<tr>
<td>E.B.I.</td>
<td>0.6</td>
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</tr>
<tr>
<td>B.B.O.</td>
<td>0.0</td>
<td>0.8</td>
</tr>
<tr>
<td>L.A.I.</td>
<td>2.4</td>
<td>0.4</td>
</tr>
<tr>
<td>H.U.M.</td>
<td>2.4</td>
<td>0.6</td>
</tr>
<tr>
<td>K.E.E.</td>
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<td>0.2</td>
</tr>
<tr>
<td>B.E.B.</td>
<td>1.3</td>
<td>0.9</td>
</tr>
<tr>
<td>K.U.T.</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>C.A.M.</td>
<td>3.8</td>
<td>1.3</td>
</tr>
<tr>
<td>N.I.C.</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>B.O.S.</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>M.E.N.</td>
<td>1.5</td>
<td>0.9</td>
</tr>
<tr>
<td>J.E.W.</td>
<td>2.0</td>
<td>0.6</td>
</tr>
<tr>
<td>E.B.B.</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>E.S.K.</td>
<td>2.1</td>
<td>1.1</td>
</tr>
<tr>
<td>C.A.Z.</td>
<td>1.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

catechol amines have not been successful so far. The results obtained were readily reproducible, and recovery of added amounts was satisfactory (80-120%). However, the amounts of the two catechol amines found in normal urine after acid hydrolysis were too high (2000-4000 µg per liter) to be acceptable without further study. Recent work by LaBrosse et al. (7) shows that normally most of the catechol amine metabolism is carried out via the o-methylation pathway. Hence, the urinary catechol amine level may not reflect a very accurate picture of its metabolism in the body.

**SUMMARY**

(1) The effect of light on the fluorescence of the ethylene diamine condensation products of the oxidized catechol amines has been studied.

(2) It was found that exposure of the norepinephrine derivative for 30 minutes to light reduced the fluorescence to a very low level. The epinephrine complex, on the other hand, is only slightly affected by the same exposure.

(3) On the basis of this differential sensitivity to light a sensitive method for the quantitative determination of the two catechol amines in blood has been developed.
(4) The levels of epinephrine and norepinephrine in the blood of normal individuals were found to range from 0.1-1.5 µg per liter and 0.3-4.0 µg per liter, respectively.

(5) Further work must be done before this method can be applied to the determination of the catechol amines in urine.

REFERENCES