A COLORIMETRIC METHOD FOR THE DETERMINATION OF ACETONE BODIES IN BLOOD AND URINE.

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The following paper describes a colorimetric method for the determination of acetone based on the reaction between acetone and salicylic aldehyde in alkaline solution with the formation of the colored product, dihydroxydibenzene acetone. The qualitative test for acetone which was introduced by Frommer in 1905 (1), based on this reaction, was modified by Engfeldt (2), who suggested a roughly quantitative method for clinical use, in which the urine is distilled and the distillate is diluted until its color with the aldehyde matches the color of one of a graded series of pure acetone solutions. In 1916 a somewhat similar method for the determination of acetone in urine was suggested by Csonka (3). The method is not adapted to the determination of small amounts of acetone. Large volumes of urine are required and the final determination is made in a colorimeter against a single standard containing forty times the minimum amount of acetone which gives a positive reaction according to Engfeldt. Like the earlier investigators Csonka used an alcoholic solution of salicylic aldehyde. His paper includes a suggestion for the determination of β-hydroxybutyric acid with the same reagents, after its oxidation to acetone by the Shaffer method. The paper does not include figures for any of the results obtained.

The present method attempts to adapt the salicylic aldehyde reaction to the determination of such small amounts of acetone as are found in normal urine and blood. It provides for the determination of 0.005 mg. of acetone in 5 cc. of distillate, so that with the dilution necessary for complete distillation a determination can be made using 2 cc. of blood, if the blood contains not less than 0.001 per cent of acetone. Greater accuracy is attained, however, if larger amounts are used.
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$\beta$-Hydroxybutyric acid is oxidized during a second distillation with potassium bichromate and sulfuric acid, as in the method first proposed by Shaffer (4) and modified by Hubbard (5). There are substances in urine which increase the color given by this second distillate in the final determination, and these are removed with copper sulfate and calcium hydroxide previous to the distillation, as suggested by Van Slyke (6). This treatment is not necessary in blood filtrates. In the procedure suggested by Csonka (3) the salicylaldehyde is added in alcoholic solution in the presence of sodium hydroxide, and the tubes are heated for 20 minutes in water kept at 45-50°C. In the present method the salicylaldehyde is added undissolved, and the tubes are heated in boiling water for from 3 to 5 minutes.

Acetone cannot be determined directly in urine or in blood filtrates with accuracy by this method. $\beta$-hydroxybutyric acid and acetoacetic acid both react to some extent under these conditions, glucose gives a distinct increase in the final color, and some urines and blood filtrates contain substances which apparently interfere with the formation of color.

The Method.

Reagents.

For the Removal of Sugar and Other Interfering Substances by the Van Slyke Method (with Modified Concentrations).—Copper sulfate, 40 per cent solution, or powdered in a mortar. Calcium hydroxide, 20 per cent suspension, or dry.

For the Oxidation of $\beta$-Hydroxybutyric Acid by the Shaffer-Hubbard Method.—50 per cent concentrated sulfuric acid. 0.2 per cent potassium bichromate solution.

For the Removal of Blood Proteins by the Folin-Wu Method.—10 per cent sodium tungstate solution. $\frac{3}{4}$ normal sulfuric acid.

For the Colorimetric Determination of Acetone.—Sodium hydroxide, 32 per cent solution. Salicylic aldehyde.

We have found great differences in the delicacy of the color reaction given by different samples of salicylaldehyde. A sample of Kahlbaum's technical salicylaldehyde proved very satisfactory, as did also Eimer and Amend's high grade product labelled "Acid salicylous, synthetic, (salicylic aldehyde)." Samples from the Eastman laboratory, though less deeply colored than the
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Eimer and Amend product, did not yield nearly so deep a color with a given amount of acetone as did the other samples used.

*Standard Acetone Solutions.*

**Stock Solution.**—This contains 0.1 mg. of acetone per cc. It is most easily prepared from a solution containing 1 cc. of acetone in 1 liter of water, whose actual acetone content by weight has been determined by an iodine titration.¹ The stock solution should be prepared from this to contain 0.1 mg. of acetone per cc. This solution can be kept for about a month without deterioration.

**Standard Solution.**—By 1:10 dilution of the stock solution a standard solution, containing 0.01 mg. per cc., is prepared for use in the actual determination. It is best to make up this dilute solution every 2nd day, and to keep it well corked when not in use.

*Determination of Acetone Bodies in Urine.*

**Preformed Acetone and Acetone from Diacetic Acid.**

(A) If β-hydroxybutyric acid is not to be determined:

Such a volume of urine as will contain about 0.1 mg. of acetone (usually from 2 to 50 cc. as required), is transferred to a 100 or 150 cc. distilling flask, the volume made up to about 75 cc. with distilled water, and 3 or 4 drops of sulfuric acid, diluted 1:1, are added. The flask is tightly fitted with a cork stopper and connected with a water-cooled condenser. The condenser is provided with a bent glass tube which has been drawn out long enough and to a sufficiently small diameter to reach to the bottom of a 25 or 50 cc. volumetric flask, and which dips below the surface of a minimum amount of water in the flask. None of the connections should be of rubber. Rubber stoppers covered with tin-foil, or cork stoppers, often renewed, can be used. The preformed acetone and acetone from diacetic acid are then distilled into the 25 or 50 cc. flask. Except when very large amounts of acetone are present a distillation to 25 cc. gives good results. When the distillate has almost reached the volume desired, the

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bent tube is disconnected and washed out with a few drops of water, and the distillate made up to volume and mixed. 5 cc. of the distillate are transferred to a test-tube and exactly 5 cc. of a 32 per cent solution of sodium hydroxide and 10 drops of salicylic aldehyde are added. Standards are prepared in test-tubes at the same time from the dilute (0.01 mg. per cc.) acetone solution. By using from 0.5 to 5 cc. of this solution a range of standards containing from 0.005 to 0.05 mg. can be made. Unless the approximate acetone content of the unknown solution is known, standards containing 0.005, 0.01, 0.02, 0.03, and 0.05 mg. had best be made. In each case the volume of the standard solution must be made to 5 cc. Exactly 5 cc. of 32 per cent sodium hydroxide and 10 drops of salicylic aldehyde are also added to each of the standard tubes. The contents of the tubes are mixed by side to side shaking and the tubes then immersed in a boiling water bath for from 3 to 5 minutes. If the salicylic aldehyde does not dissolve easily the tubes must be shaken until solution is effected. After the heating the tubes are removed and allowed to cool, the solutions filtered, and colorimetric comparison is made. The standard used should be of such a concentration that the unknown solution gives a reading between 11 and 19 mm. with the standard set at 15 mm.

In making the calculation the following formula may be used:

\[
\frac{x}{y} \times \frac{p}{s} \times \frac{t}{5} \times 100 = \left\{ \begin{array}{l}
\text{No. of gm. of acetone in 100 cc. of blood or urine.}
\end{array} \right.
\]

where:

- \( x \) = reading of standard.
- \( y \) = reading of unknown.
- \( p \) = gm. acetone in standard.
- \( s \) = cc. of blood or urine used.
- \( t \) = cc., volume of total distillate.

(B) If \( \beta \)-hydroxybutyric acid is also to be determined:

If \( \beta \)-hydroxybutyric acid is to be determined in addition to acetone, sugar and other interfering substances must be removed, even from normal urine, before any distillation is made. For this the urine is treated with copper sulfate and calcium hydroxide according to the Van Slyke procedure (6). In order to keep down the volume of solution to be distilled, however, the urine is diluted 1:5 instead of 1:10, using 1 volume of urine, 1 volume of 40 per cent copper sulfate solution, and enough of a 20 per...
cent suspension of calcium hydroxide to make the reaction alkaline to litmus (probably 1 volume). The whole mixture is then made up to 5 volumes. If the urine is very low in acetone bodies the copper sulfate can be powdered in a mortar and both this and the calcium hydroxide added in powdered form. The copper sulfate should be dissolved before the calcium hydroxide is added. The mixture must be shaken very thoroughly and allowed to stand for ½ or ¾ of an hour, with occasional shaking. It is then filtered and a volume of the filtrate equivalent to from 2 to 50 cc. of urine (depending on the acetone content) placed in a 300 cc. distilling flask and made acid with 3 or 4 drops of sulfuric acid (diluted 1:1). The volume is made up to about 75 cc., the flask fitted with a dropping funnel, and connected with a water-cooled condenser, and the distillation and determination of acetone and diacetic acid carried out as described under (A).

Oxidation of β-Hydroxybutyric Acid by the Hubbard-Shaffer Method and Its Determination as Acetone.

After distillation of the preformed acetone, a 100 cc. receiving flask is substituted for the 25 cc. flask, the residue in the distilling flask is brought to a boil, and 30 cc. of half concentrated sulfuric acid and 20 cc. of 0.2 per cent potassium bichromate are added gradually through the dropping funnel while a slow distillation goes on. 50 cc. more of the bichromate are added after 10 minutes and 50 cc. more after another interval of 10 minutes. The process differs from the Hubbard method only in that the distillation is made very slowly and the volume of distillate kept down to 100 cc. The distillation should occupy at least 30 minutes. When the distillation to 100 cc. is almost complete the receiving apparatus is again disconnected, the bent tube washed down with a little water, and the distillate made up to 100 cc. and mixed. Acetone is determined colorimetrically in 5 cc. of the distillate as described above under (A).

Determination of Acetone Bodies in Blood.

Preformed Acetone and Acetone from Diacetic Acid.

The blood proteins are precipitated by the regular Folin-Wu method (7), making a dilution of the blood of 1:10. From 10
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to 100 cc. of the filtrate, depending on the acetone content, are transferred to a 300 cc. distilling flask, 3 or 4 drops of concentrated sulfuric acid, diluted 1:1, added, the volume made up to 50 to 75 cc., and distillation carried out as described for the determination in urine. The distillation is made into a 20 cc. receiving flask or graduated test-tube unless the acetone content of the amount of blood used is known to be high (above 0.05 mg.), in which case the distillation is made into a 25 cc. flask or graduated tube. If the amount of acetone in the filtrate used is known to be 0.1 mg., or more, the distillation may be made to 50 cc. In any case the distillation is stopped just before the desired volume has been reached, the distillate is made up to volume, and 5 cc. of the distillate are heated with alkali and salicylic aldehyde, cooled, and read in a colorimeter as described for the urinary determination.

**Determination of Acetone from β-Hydroxybutyric Acid in the Blood.**

1. If the actual volume of filtrate used is known to contain 0.1 mg. of β-hydroxybutyric acid as acetone, or more, the distillation is carried out as described for the determination in urine, the volume of the distillate being kept within 100 cc., and finally made up to 100 cc., and the colorimetric determination being made upon 5 cc. of the distillate, as described above under (A).

2. If the volume of filtrate used is expected to contain less than 0.1 mg., the distillation is carried out without regard to the volume of distillate collected, and after the 30 minute period, the distillate is redistilled into a 20, 25, or 30 cc. volumetric flask (or graduated test-tube), according to the amount of acetone expected. In any case the distillate is made up to volume and the acetone content of 5 cc. of the distillate determined as described above.

The formula given in the section on urinary determination under (A) may be used for all the calculations.

Tables I and II give figures for the recovery of acetone and β-hydroxybutyric acid from urine and blood by the new method and Table III gives figures for acetone by the new method as compared with figures given by the iodometric method.

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**TABLE I.**

Recovery, by the New Method, of Acetone and β-Hydroxybutyric Acid Added to Urine.

All results are expressed as mg. of acetone per 100 cc. of urine.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Preformed acetone and diacetic acid from the urine</th>
<th>Added acetone recovered</th>
<th>β-hydroxybutyric acid from the urine</th>
<th>β-hydroxybutyric acid added</th>
<th>Added β-hydroxybutyric acid recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal human</td>
<td>0.13</td>
<td>0.25</td>
<td>0.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>0.18</td>
<td>1.00</td>
<td>1.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>0.10</td>
<td>1.00</td>
<td>0.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>0.43</td>
<td>1.00</td>
<td>0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>0.45</td>
<td>2.00</td>
<td>1.55</td>
<td>1.70</td>
<td>4.56</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.38</td>
<td>3.77</td>
<td>3.48</td>
<td>1.97</td>
<td>9.12</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.40</td>
<td>3.77</td>
<td>3.64</td>
<td>0.74</td>
<td>9.12</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.50</td>
<td>3.77</td>
<td>3.84</td>
<td>3.20</td>
<td>8.00</td>
</tr>
<tr>
<td>Diabetic human*</td>
<td>3.12</td>
<td>2.00</td>
<td>1.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog (phlorhizinized)</td>
<td>19.60</td>
<td>7.54</td>
<td>7.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*We are indebted to Dr. Edward Tolstoi for supplying us with diabetic urines and bloods.

**TABLE II.**

Recovery, by the New Method, of Acetone and β-Hydroxybutyric Acid Added to Blood.

All results are expressed as mg. of acetone per 100 cc. of blood.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Preformed acetone and diacetic acid from blood</th>
<th>Added acetone recovered</th>
<th>β-hydroxybutyric acid from the blood</th>
<th>β-hydroxybutyric acid added</th>
<th>Added β-hydroxybutyric acid recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef</td>
<td>0.08</td>
<td>0.075</td>
<td>0.000</td>
<td>1.80</td>
<td>7.91</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.11</td>
<td>0.075</td>
<td>0.074</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>0.21</td>
<td>0.754</td>
<td>0.910</td>
<td>1.86</td>
<td>0.47</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.32</td>
<td>0.188</td>
<td>0.170</td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.33</td>
<td>0.377</td>
<td>0.347</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog (phlorhizinized)</td>
<td>3.24</td>
<td>1.88</td>
<td>1.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>6.15</td>
<td>0.75</td>
<td>1.01</td>
<td>6.86</td>
<td>4.00</td>
</tr>
<tr>
<td>&quot;</td>
<td>6.15</td>
<td>11.31</td>
<td>11.23</td>
<td>6.86</td>
<td>8.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.14</td>
</tr>
</tbody>
</table>
TABLE III.

Comparison of Urinary Acetone in the Same Distillate by Iodine Titration and Salicylic Aldehyde Determination.

Expressed as mg. of acetone per 100 cc. of urine.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>By iodine titration</th>
<th>By salicylic aldehyde reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog (phlorhizinized)</td>
<td>13.50</td>
<td>13.30</td>
</tr>
<tr>
<td></td>
<td>19.52</td>
<td>19.07</td>
</tr>
<tr>
<td></td>
<td>20.30</td>
<td>19.80</td>
</tr>
<tr>
<td></td>
<td>20.40</td>
<td>19.45</td>
</tr>
<tr>
<td></td>
<td>26.92</td>
<td>26.39</td>
</tr>
<tr>
<td></td>
<td>30.45</td>
<td>30.90</td>
</tr>
<tr>
<td>Human diabetic</td>
<td>16.90</td>
<td>16.70</td>
</tr>
<tr>
<td></td>
<td>18.80</td>
<td>17.56</td>
</tr>
<tr>
<td></td>
<td>27.84</td>
<td>27.89</td>
</tr>
</tbody>
</table>

SUMMARY.

A colorimetric method is described for the determination of the acetone bodies in normal or pathological urine or blood, based on the reaction of acetone with salicylic aldehyde in alkaline solution.

BIBLIOGRAPHY.

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