A MODIFIED SALICYLALDEHYDE METHOD FOR THE DETERMINATION OF ACETONE BODIES IN BLOOD AND URINE

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(Received for publication, July 3, 1940)

Colorimetric methods for the determination of acetone based on the reaction of acetone with salicylaldehyde in alkaline solution hold an intermediate position, with respect to specificity and sensitivity, between gravimetric and iodometric methods (1). In these respects they appear to have no advantage over recently published methods (2, 3) which combine the specificity of mercury precipitation with a high degree of sensitivity. However, because of their greater rapidity and simplicity of technique salicylaldehyde methods may frequently be preferred.

In previous salicylaldehyde methods use has been made of a boiling water bath (4, 5), or of a bath at 45–50° (6, 7), to hasten color development. The former procedure has been criticized (7) because of the loss of small amounts of acetone at this high temperature, while the latter procedure is slower and less convenient. In the present paper a modification is proposed which reduces the time necessary for the development of color without the application of heat. The speed with which the salicylic aldehyde reaction takes place depends upon the concentration of reacting substances as well as upon temperature. If the concentration of reagents exceeds a certain point, a precipitate is formed which has hitherto been a limiting factor. In the present method the concentration of the reacting mixture is increased and the precipitate which forms is dissolved after the reaction is complete.

Preliminary Procedures The colorimetric determination of acetone, preformed or from the other acetone bodies, is made on a distillate from blood filtrate or urine. Except in the determination of urinary acetone and diacetic acid the sensitivity of the method
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is limited by the procedures involved in the preparation of these distillates. These procedures, as slightly modified from those previously used (4, 5), are briefly as follows:

An all-glass distilling apparatus is recommended, especially for the determination of β-hydroxybutyric acid. This is not essential and well fitting corks, but no rubber, may be used for connections. The apparatus consists of a 200 to 300 cc. distilling flask with dropping funnel, a 200 mm. water-cooled condenser and delivery tube, and a receiving tube or flask for which it is convenient to use a 15 cc. graduated centrifuge tube for acetone and diacetic acid and a 200 cc. round bottomed flask for β-hydroxybutyric acid. The enlarged part of the delivery tube should rest in the top of the receiver, forming a cover for it, and the lower end is drawn out to a fine tip which reaches to the bottom of the receiver, where it is covered by a minimum amount of water. Glass beads in the distilling flask and a microburner, with no sand bath, are used. Glass joints are lubricated with water. Heat is applied slowly at the beginning of distillation to prevent violent bubbling in the receiving tube.

For the determination of acetone and diacetic acid a volume of blood filtrate or urine (15 to 30 cc.) is acidified with sulfuric acid and distilled to a volume of distillate equal to one-third, or more, of the original volume.

For the determination of β-hydroxybutyric acid in urine interfering substances are removed by the Van Slyke copper sulfate-calcium hydroxide procedure (8). All volumes may be reduced proportionally if advisable. If the urine is dilute, its volume may be increased in relation to the volume of the final mixture, twice the amount of copper sulfate (in 40 per cent solution) and calcium hydroxide being used. After standing for 30 to 45 minutes the mixture is filtered, or centrifuged and filtered. An aliquot of the filtrate is transferred to the distilling flask and acidified with sulfuric acid. Water is added, if necessary, to make the volume not less than 30 cc. The dropping funnel is inserted and one-third, or more, of the volume is distilled to remove acetone and diacetic acid, leaving a volume of 20 to 60 cc. (approximately) in the distilling flask. Since some acetone is lost during the copper-calcium treatment, a separate distillation should be made

1 An apparatus of this sort, with interchangeable ground glass joints, has been made for the writer by Eck and Krebs, New York.
from untreated urine to determine acetone and diacetic acid. It has been found possible to reduce the time for oxidation and distillation of $\beta$-hydroxybutyric acid to half that prescribed by the Hubbard method\(^2\) (9). This alteration reduces the volume of distillate and consequently increases the sensitivity of the method. The solution is brought to a boil and 15 cc. of sulfuric acid (concentrated, diluted 1:1) and 10 cc. of 0.2 per cent potassium dichromate are added by drops during the first 5 minutes of distillation, followed by 25 cc. of the dichromate during each of the following 5 minute periods. The rate of distillation is regulated so that from about 50 to 85 cc. are distilled at the end of 15 minutes.

$\beta$-Hydroxybutyric acid in blood filtrate is determined in the same way except that the copper-calcium treatment and separate distillation of acetone and diacetic acid are unnecessary.

**Colorimetric Determination**


Standard acetone solutions. As previously described (4). For accuracy the stock solution should be standardized by iodine titration. For less accurate work it can be assumed that 5 cc. of freshly opened or well preserved c.p. acetone diluted to 500 cc. give a 0.78 to 0.79 per cent solution. Artificial standards are described in Table I.

Ethyl alcohol. (For Procedure B only.) Aqueous 70 to 75 per cent solution.

*Procedure*—Exactly 0.1 cc. of salicylaldehyde is measured into a tube graduated to 5 and 10 cc.\(^3\) with a 0.1 cc. pipette\(^4\) or a

\(^2\) The dichromate-sulfuric acid procedure, as applied in our methods, is not entirely satisfactory for the determination of small amounts of $\beta$-hydroxybutyric acid. More sensitive methods for this oxidation are at present being investigated.

\(^3\) Tubes made by the Klett Manufacturing Company, Inc., New York, for use in the Klett-Summerson photoelectric colorimeter, graduated to 5 and 10 cc., are particularly convenient. Tubes graduated to 10 cc., of uniform bore, made by the Fales Chemical Company, Cornwall Landing, New York, for use in the Kingsbury-Clark albumin determination, are also convenient.

\(^4\) A short, 0.1 cc. pipette made by Eimer and Amend for the Folin micro
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1 cc. pipette graduated to tenths (slightly less accurate), followed by 2 cc. of the distillate or acetone standard and 1.5 cc. of the potassium hydroxide from a burette with a fine glass tip. The contents of the tube are mixed by several churning motions with a glass rod flattened at one end at right angles to the rod to form a foot of a size to fit easily into the tubes used. The rod is left in the tube which is allowed to stand at room temperature for 20 minutes or longer. After this the precipitate is dissolved by adding either (Procedure A) distilled water to 10 cc. (or more, see below), or (Procedure B) the aqueous alcohol to 5 or to 10 cc. (see below). Solution and mixture are accomplished by churning up and down briskly with the rod. 

Choice between Procedures A and B depends largely upon the acetone concentration, since Procedure B gives more, and Procedure A less, color. The use of both solvents widens the range of acetone concentrations which can be determined, but for certain types of work it may be satisfactory to use either one or the other exclusively. The range of concentration for which each solvent is practical is discussed in the following paragraph. During the period of color development it is possible to estimate roughly how much acetone is present and therefore which solvent is preferable. Since the two ranges overlap, the choice is significant only for very low and very high concentrations. The reading may be made in either a visual or a photoelectric colorimeter. With the former, acetone standards (or artificial standards), as described below, are used and the calculation is made as usual. Micro plungers and cups are convenient for use with Procedure B. With a photoelectric colorimeter a curve for comparison may be constructed from standardized acetone solutions. For approximate results a side-to-side comparison may be made in test-tubes of uniform bore, with the artificial standards described below. 

After Procedure A the color blood sugar method has been found particularly convenient for these measurements.

Potassium instead of sodium hydroxide is used in the present method because its greater solubility makes it possible to obtain a higher concentration of alkali during the reaction. Aside from this it appears to have no specific advantage over sodium hydroxide.

The Klett-Summerson photoelectric colorimeter has been found satisfactory for this determination which can be carried out entirely in the standard tubes made for this instrument.
fades, and after Procedure B there is a slight and gradual increase in color. For this reason, if acetone standards are used, they should be diluted at the same time as the unknowns. If artificial standards or a photoelectric colorimeter is used readings should be made within 15 minutes after dilution by Procedure A and within 30 minutes after dilution by Procedure B.

**Range of Concentration and Proportionality**—Solutions which contain from about 0.5 to 20 mg. per cent of acetone\(^*\) can be read in a visual calorimeter after addition of water to 10 cc. (Procedure A). With this instrument and procedure an exact indirect proportionality exists between acetone concentration and scale reading for all concentrations above 1 mg. per cent. Below this the readings are exact for concentrations between 0.5 and 0.75 and between 0.75 and 1.0 mg. per cent. Concentrations too dark to be read can be further diluted with water, after the reaction has taken place, so that the color falls within the reading range. The reagents used in the method are sufficient to react with 2 cc. of a 200 mg. per cent acetone solution, and dilution can be made to as much as 200 cc. without disturbing the proportionality of the reading against standards diluted to only 10 cc., provided the color determined is not less than that given by a 2 mg. per cent solution. With Procedure B concentrations of from about 0.05 to 5 mg. per cent of acetone can be read in a visual calorimeter if the volume is made to 5 cc. and a little over twice these amounts if the volume is made to 10 cc. Readings are correctly proportional to concentration if the latter is above about 0.25 mg. per cent. Below this concentration the range of proportionality is limited. Concentrations of over 0.25 mg. per cent, made to 10 cc., can be read against those made to 5 cc. With a photoelectric colorimeter the reading range depends upon the type of instrument and filter used. With a Klett-Summerson colorimeter and green filter (No. 54) the range of acetone solutions which can be read is from about 0.02 to 8 mg. per cent after water dilution to 10 cc. and from about 0.01 to 1.5 mg. per cent after alcohol dilution to 5 cc. The curve obtained by plotting acetone concentration against the logarithmic scale readings of this instrument deviates from a straight line.

\(^*\) The figures given in connection with concentration refer to the mg. of acetone in 100 cc. of the solution or distillate, 2 cc. of which are used in the determination. The amount of acetone present during the determination is, in each case, one-fiftieth of this figure.
after Procedure A, the increase in readings becoming smaller with increasing concentration. Practically a straight line curve is obtained after Procedure B (solution made to 5 cc.) between 0.1 and 1.5 mg. per cent concentrations. A more satisfactory filter might be found for this determination. The blue and red filters which have been tried were not found suitable.

Choice of Acetone Standards—If acetone standards are used, there is considerable latitude in the choice of those to be made with each set of determinations, because of the proportionality which exists between concentration and depth of color, and because of the possibility of additional dilution of either standard or unknown after the reaction has taken place. If both Procedures A and B are used, the following scheme is suggested. 0.1, 1.0, and 5.0 mg. per cent acetone solutions are made up from the stock solution, and five standard tubes are prepared from them. 1 cc. of the 0.1 mg. per cent solution and 1 cc. of distilled water are put into Tube 1, 2 cc. of the same solution into Tube 2, 2 cc. of the 1.0 mg. per cent solution into each of Tubes 3 and 4, and 2 cc. of the 5 mg. per cent solution into Tube 5. The tubes are treated according to the directions given above for the method. After the reaction has taken place, Tubes 1 and 2 are made to 5 cc., and Tube 3 to 10 cc., with the alcohol solution; Tubes 4 and 5 are made to 10 cc. with distilled water. The alcohol standards are equivalent to 0.05, 0.1, and 0.5, and the water standards to 1.0 and 5.0 mg. per cent solutions. Unknowns of from 0.05 to 200 mg. per cent can be read against these standards. Unknowns obviously below 1.0 mg. per cent can be read against each solvent. Each is read against the nearest standard dissolved by the same solvent. Unknowns of concentrations over 1.0 mg. per cent dissolved with alcohol and those of over 10 mg. per cent dissolved with water can be further diluted with the same solvent to come within the range of the 0.5 and of the 5.0 mg. per cent standards. By this scheme the scale readings are correctly proportional to the concentration for all unknowns above 0.25 mg. per cent diluted with alcohol, and above 0.75 mg. per cent diluted with water. Below these amounts there may be an error of from 5 to 10 per cent of the concentration.
Artificial color standards have been made for use after Procedure A. The value of such standards is limited by possible variations in the chromogenic capacity of different samples of salicylaldehyde, as previously discussed (10). However, in the past 9 years the writer has obtained over a dozen different samples of the Eimer and Amend product at different times, all of which gave the same color in this reaction, so that the use of artificial standards appears justified as long as this product is available. For much experimental, as well as routine, work, when great accuracy is not demanded, these standards are useful and time-saving. The standards described in Table I were made to match acetone solutions, standardized by iodine titration and treated according to this method. They can be used for side-to-side comparisons, by transmitted light, in test-tubes of a uniform bore of approximately 13 mm., or for comparison in a visual colorimeter, with the standard set at 13 mm. They were made to match acetone solutions at this depth because this is the approximate diameter of both the Fales and Klett, uniform bore tubes which have been used in the side-to-side comparison. In a visual colorimeter the proportionality with these standards does not cover as wide a range as with acetone solutions. They have no value in a photoelectric colorimeter.

### Table I

**Directions for Making Artificial Standards for Use after Procedure A**

Only c.p. products should be used. The amounts specified are made to 100 cc. with distilled water. All standards except the last two may be prepared from 10 per cent solutions of the dichromate and cobalt chloride.

<table>
<thead>
<tr>
<th>Acetone equivalent (mg. per cent)</th>
<th>Potassium dichromate (gm.)</th>
<th>Cobalt chloride (gm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.065</td>
<td>0.64</td>
</tr>
<tr>
<td>1.0</td>
<td>0.095</td>
<td>1.40</td>
</tr>
<tr>
<td>2.0</td>
<td>0.60</td>
<td>1.80</td>
</tr>
<tr>
<td>3.0</td>
<td>1.80</td>
<td>1.70</td>
</tr>
<tr>
<td>4.0</td>
<td>3.30</td>
<td>1.60</td>
</tr>
<tr>
<td>5.0</td>
<td>5.00</td>
<td>1.50</td>
</tr>
<tr>
<td>7.5</td>
<td>13.30</td>
<td>0.80</td>
</tr>
<tr>
<td>10.0</td>
<td>13.50</td>
<td>0.30 + 8 cc. concentrated sulfuric acid</td>
</tr>
</tbody>
</table>
The Behre and Benedict (4) and the Behre (5) methods are subject to the criticism of Ravin (7) that traces of acetone are lost when a boiling water bath is used for the development of color, as evidenced by the qualitative test (11). Ravin also pointed out that the addition of salicylaldehyde by drops is subject to inaccuracy. The present method, to which these objections are not applicable, is felt to be preferable to either of the earlier ones, but it might be stated that in the Behre and Benedict method these inaccuracies can be greatly reduced by careful and uniform technique and that in the Behre clinical method they are insignificant because of the purpose of the test and the type of colorimetric comparison used.

At least two reactions appear to be involved in the formation of maximum color with salicylaldehyde; one, which gives most of the color, is hastened by warming, while the other, which gives an additional fraction of color, is inhibited as the temperature is raised. This secondary color appears when solutions are allowed to cool after warming and almost entirely disappears if they are rewarmed. “Blank” color from the reagents alone increases on cooling but does not account for the total increase. The full color from both reactions is developed slowly at room temperature, or if the solutions are chilled to 10° and then allowed to stand at room temperature. The secondary color which develops on cooling was discussed by Ravin (7) in whose method a 30 minute cooling period is used to develop maximum color. Korenman’s method (6) is similar in this respect. Behre and Benedict recognized this increase in color on cooling, but sacrificed maximum color to speed. In the present method the temperature in the tubes is raised to about 37-40° by the addition of the concentrated alkali, and drops almost to room temperature during the 20 minute standing period. The spontaneous warming and cooling favor the development of maximum color.

If all tubes in a series are treated exactly alike in this method, and if standard and unknown are of nearly the same concentration, the error due to loss of acetone is very slight, and, if the same number of drops of salicylaldehyde is added to each of a series of tubes in succession, from a long pipette held in a semihorizontal position, uniform results can be obtained in any one series.
The specificity of the salicylaldehyde reaction for acetone has been studied by Braunstein (12) and Thomson (13). The latter author found the reaction more sensitive for acetone than for any of the other substances studied which gave a positive reaction. These included acetaldehyde, pyruvic acid, propionic acid, ethyl acetoacetate, and a number of other compounds. It is found that with the method described in this paper acetaldehyde, pyruvic acid, and lactic acid, in amounts occurring in biological fluids, do not interfere. A 25 mg. per cent solution of acetaldehyde gives approximately the same amount of color as a 0.2 mg. per cent acetone solution, while a 500 mg. per cent acetaldehyde solution corresponds to about 25 mg. per cent of acetone. The acetaldehyde color is yellower, less red, than the acetone color, which makes exact comparisons difficult. Pyruvic acid reacts to give about one-fiftieth as much color as acetone (the color is also yellower), but distillates from acetone and pyruvic acid show no effects from the latter. The same is true of lactic acid, which does not itself react. However, if 25 mg. of pyruvic acid are oxidized and distilled by the β-hydroxybutyric acid procedure, the distillate gives a color with salicylaldehyde equivalent to that of about 0.4 mg. per cent of acetone. The distillate from a large excess of lactic acid (600 mg.) treated in the same way gives a yellower color with salicylaldehyde than does a blank. The study of possible interference from other biological substances is being continued.

The writer wishes to acknowledge with gratitude the assistance of Professor Chester J. Farmer and Dr. Smith Freeman of Northwestern University Medical School, and of Dr. William Muhlberg and Mr. George O'Connor of the Union Central Life Insurance Company.

SUMMARY

A modified salicylaldehyde method for the determination of acetone in distillates from urine or blood filtrate is described. The reaction takes place in a concentrated mixture of the reacting substances without application of heat and is complete in 20 minutes. The precipitate which forms is dissolved either in water or alcohol. The range and sensitivity of the method are somewhat increased over that of previous salicylaldehyde methods. Either
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a visual or photoelectric colorimeter can be used. An almost exact proportionality exists between acetone concentration and scale reading in a visual colorimeter. Artificial color standards for visual colorimetry are also described. Procedures for the oxidation of β-hydroxybutyric acid and the distillation of acetone have been slightly modified. The specificity of the reaction is briefly discussed.

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