Determination of Ketone Bodies in Blood and Urine by Means of Vanillin in Alkaline Medium

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In a previous communication (1) we have reported that vanillin reacts with a large number of organic compounds with a carbonyl group, with aldehydes, and particularly with ketones, diketones, and ketonic acids to produce yellowish, or yellowish-red color complexes. Because of its wide reactivity the vanillin-alkali reagent cannot be used for any specific carbonyl compound unless it is first separated by extraction with solvents, by precipitation, by chromatography, by electrophoresis, or by distillation or aeration especially if the compound has a low boiling point. Acetone has a low boiling point, 56.5°C. It can therefore be separated easily by aeration or distillation from other compounds in a biologic fluid or solid tissue. Acetoacetic acid may also be determined, for upon treatment with acid it is readily converted to acetone. The ketogenic acid, β-hydroxybutyric acid, also yields acetoacetic acid on oxidation. We have developed a method for acetone based upon its separation from blood and urine by distillation and subsequent treatment of the distillate with a vanillin-alkali reagent. This method offers many advantages over the very widely used procedure of Behre and Benedict (2) in which salicylaldehyde in alkaline medium is utilized as the reagent.

Salicylaldehyde or 2-hydroxybenzaldehyde is a compound similar in chemical reactivity to vanillin or 3-methoxy-4-hydroxybenzaldehyde. The formation of a reddish-colored complex, the sodium salt of

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dihydroxydibenzalacetone, resulting from the interaction of acetone and salicylaldehyde was first reported by Fabinyi (3) in 1901. This reaction was later utilized by Rosenthaler (4), Frommer (5), and Leffmann (6) as a qualitative test for acetone in urine, and by Deshons (7) for acetone in spinal fluid.

The salicylaldehyde-alkali reaction was first suggested by Czonka (8), in 1916, as a quantitative method for acetone. Behre and Benedict (2), in 1926, reported a similar method for ketone bodies. Ravin (9), in 1936, published a criticism of the Behre and Benedict procedure and proposed modifications to overcome the disadvantages of their technic, and in 1940, Behre (10) modified the original Behre and Benedict procedure to overcome the objections raised by Ravin.

Vanillin (3-methoxy-4-hydroxybenzaldehyde) is a compound possessing many chemical properties in common with salicylaldehyde. Both are aromatic aldehydes and both possess a phenol group. Vanillin possesses several important advantages over salicylaldehyde. The former is a white crystalline compound that can be weighed accurately. It is fairly soluble in water, but not too soluble to prevent its purification by crystallization from water. Salicylaldehyde is a liquid, insoluble in water, deteriorating very rapidly and developing a deep brown color on standing even for a short time in a brown bottle. For use as a reagent it requires purification by distillation. Behre and Benedict (2) found great differences in the delicacy of the color given by different samples of salicylaldehyde reacting with acetone. Ravin (9) and Nadeau (11) also noted marked variations in the intensity of the color produced by different brands of salicylaldehyde. Furthermore, to be utilized as a qualitative or quantitative reagent for acetone, salicylaldehyde must be dissolved in alcohol. The use of alcohol as a solvent complicates matters, unless the alcohol is also purified to remove carbonyl-containing compounds by means of silver salts (silver acetate) and alkali (sodium hydroxide), or better still by means of 2,4-dinitrophenylhydrazine subsequent to distillation. Such carbonyl-bearing compounds also produce colored complexes with salicylaldehyde as well as with vanillin in alkaline medium.

Vanillin is more stable, although, according to Englis and Manchester (12), a dilute solution of vanillin (10 Gm. per liter in 10 per cent ethyl alcohol) is slowly oxidized to vanilllic acid, the oxidation being fairly complete in several weeks. Solutions of vanillin do not darken on standing. Fresh solutions, if need be, can be readily and quickly prepared. Concentrated alkali added to salicylaldehyde pro-
duces a yellow color at room temperature. The yellow characteristic color produced by acetone and salicylaldehyde with concentrated alkali is necessarily an addition to the color produced by salicylaldehyde and alkali alone. Vanillin solutions in the cold do not take on color upon the addition of alkali. Many investigators have utilized vanillin as a reagent in a medium made acid with more or less concentrated solutions of sulfuric acid. Vanillin yields a characteristic yellow color with sulfuric acid without the aid of heat. Nikitin (13) made a comparison of the reactivity of acetone with vanillin and with salicylaldehyde in equal concentrations of alkali. He found vanillin to be 5.27 times more reactive than salicylaldehyde.

Of the 103 compounds—aliphatic, alkyl-aryl, and aryl ketones, cyclic ketones, ketone carboxylic acids, aliphatic and aromatic aldehydes, aldos, ketols, acetols, and some α-hydroxyacids—we have investigated with the vanillin-alkali reagent, acetone proved to be the most sensitive and the most intensely reactive (1).

While neither the salicylaldehyde-alkali reaction nor the vanillin-alkali reaction is specific for acetone, we can take advantage of its relatively low boiling point (56.5°) and volatility to separate it from other reacting compounds. Removal of the acetone from biologic fluids is necessary because of the interfering color development with alkali alone or with the vanillin-alkali reagent. A urine sample was acidified with sulfuric acid and boiled until the sample was free of acetone and acetoacetic acid. When cooled the heated urine was treated with the vanillin-alkaline reagent. The reaction mixture turned light brown. Addition of alkali alone produced the same result as heating. Furthermore, certain nonvolatile compounds, such as pyruvic acid and other α-ketonic acids, also react with vanillin in alkaline medium (1). Pyruvic acid also reacts with the salicylaldehyde-alkali reagent (14).

REACTION OF VANILLIN WITH ACETONE

Vanillin condenses with acetone to form vanillalacetone or divanillalacetone. Glaser and Tramer (15) prepared the sodium salt of vanillalacetone by treating vanillin and acetone in alkaline medium. The sodium salt crystallized as yellow needles. Vanillalacetone itself formed beautiful broad yellow-white needles melting at 129°. From a medium acidified with hydrochloric acid Glaser and Tramer prepared divanillalacetone as reddish-yellow or orange crystals, which
lost water of crystallization at 100°. The anhydrous compound formed green prismatic crystals melting at 155-160°.

\[
\begin{align*}
\text{vanillin} & \quad \rightarrow \quad \text{vanillalacetone} \\
\text{vanillin} & \quad \rightarrow \quad \text{divanillalacetone}
\end{align*}
\]

**QUALITATIVE METHOD**

Acetone (plus diacetic acid) can be qualitatively detected in urine or blood by means of a procedure similar to the one adopted by Behre (10). Instead of her reagent, salicylaldehyde, we have substituted vanillin. In our procedure 5 ml. of urine or protein-free blood are placed in a test tube and 1 drop of 1:1 sulfuric acid added. A small thin square of cotton is treated in the center with one drop of 1% vanillin solution and two drops of 10N sodium hydroxide solution. The cotton square is inserted in the test tube with the spot reagent facing downward. The tube is placed upright in boiling water for 5 minutes. In the presence of the ketone bodies, acetone, or diacetic acid, the reagent in the cotton plug assumes a yellow or yellowish-red or reddish color depending upon the quantity of ketone bodies present.

Because of the high volatility of acetone, it was thought possible that the Conway microdiffusion technic (16) could be adopted for the detection of acetone. Such a diffusion method for acetone has been described by Werch (17). Acetone solutions as well as urines con-
taining definite amounts of acetone were introduced into the outer chamber of the Conway unit. Into the center of the Conway unit were placed 1 ml. of vanillin solution and 2 ml. of 40% sodium hydroxide. The lid of the unit was sealed with petroleum jelly, and the unit incubated at 37° for two hours. Using acetone standards of widely varying concentrations the mixture in the inner well containing the vanillin-alkali reagent developed a pale orange color. After 24 hours with comparatively larger concentrations of acetone, long reddish needles crystallized out. The Conway diffusion technic with the vanillin-alkali reagent has the possibility of being worked out as a quantitative method for acetone in biologic fluids. Winnick (18) has developed a diffusion method for acetone in blood and urine based on the Conway diffusion procedure. The acetone is trapped in sodium bisulfite, liberated from the aldehyde-sulfite addition product and titrated with 0.005N iodine. Thin and Robertson (19) recently developed a diffusion method for the ketone bodies, utilizing salicylaldehyde and 4N potassium hydroxide as the reagents.

**QUANTITATIVE METHOD**

Nikitin and Vershinsky (20), in 1937, reported a quantitative method for acetone by means of vanillin in a highly concentrated alkaline solution. Their method, published in Russian, was not developed specifically to meet biologic requirements, and is not a spectrophotometric method. In order to develop a quantitative biochemical procedure of a photometric nature we must determine: (1) the sensitivity of the reaction, (2) the absorption spectrum in order to set the photometric apparatus at the proper wave length as indicated by the peak in the absorption spectrum, (3) the ability of the reaction system to follow the Beer-Lambert law, and (4) the extent of recovery of a definite quantity of the compound added to biologic material.

We have found the vanillin-alkali reaction for acetone to be sensitive to 2 μg. Figure 1 gives the absorption spectrum with a peak at 420 μm. The calibration curve in Fig. 2 is indicative of the fact that the reaction follows the Beer-Lambert law. Table 1 shows the percentage of recoveries of added quantities of acetone from blood and from urine.

**REAGENTS**

All the reagents should be made up with doubly distilled water free from the iron and copper compounds, which act as catalysts of oxida-
Fig. 1. Absorption spectrum of acetone.

Table 1. Analysis of Blood and Urine

<table>
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<tr>
<th></th>
<th>Acetone and diacetic acid* (mg./100 ml.)</th>
<th>Acetone added (mg./100 ml.)</th>
<th>Acetone found (mg.)</th>
<th>Acetone recovered (mg.)</th>
<th>% Recovered</th>
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<td>5.00</td>
<td>5.65</td>
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*Measured as acetone.
The distilled water should be freshly boiled and cooled to room temperature. The precaution of boiling is to remove oxygen which may be dissolved in the water in order to minimize the oxidation of vanillin to vanillic acid. These precautions improve the keeping qualities of the vanillin reagent.

I. Vanillin reagent. Vanillin (Eastman-Kodak) is recrystallized from hot distilled water. With the dry crystals a solution of 0.72% vanillin is prepared. This solution corresponds to a 0.047 molar solution of vanillin. We have found that a 1% solution of vanillin tends partially to crystallize out on standing.

The vanillin should be kept in a brown bottle with a glass stopper. We have tested the reagent after a month and found that it had not deteriorated during that length of time.
2. Sodium hydroxide (10N). This solution is prepared by dissolving 400 Gm. of C.P. sodium hydroxide pellets in cooled freshly distilled water and diluted to 1 liter.

3. Sulfuric acid. 1:1 sulfuric acid, reagent grade.

4. Sodium bisulfite. A freshly prepared 2.0% solution made from analytic grade reagent.

5. Acetone, purified by distillation, if necessary.

To standardize acetone, 5 ml. acetone solution are placed in a 250 ml. glass-stoppered flask. At first 25 ml. 1N sodium hydroxide solution are added and then 50 ml. 0.1N iodine solution. This solution should be measured with great accuracy. The iodine solution is added a little at a time and the container shaken after each addition. The flask is stoppered and allowed to stand for 10 to 15 minutes. Normal sulfuric acid is added to the extent of 26 to 30 ml. In order to liberate iodine, the acid must be in excess of the alkali present in the mixture. Acetone reacts with the iodine to form iodoform. Excess iodine is titrated with 0.1N sodium thiosulfate, using starch solution as indicator. The quantity of 0.1N iodine originally added minus the quantity of unused 0.1N iodine equals the quantity of 0.1N iodine that reacted with acetone. One milliliter of 0.1N iodine is equivalent to 0.9675 mg. acetone.

A standard acetone stock solution is made up so as to contain 1 mg. of acetone per milliliter. This standard stock solution should be kept in the refrigerator in a glass-stoppered bottle. It does not deteriorate for a month. Freshly prepared dilute standards are made by diluting 0.1 ml., 0.5 ml., 1.0 ml., and 5 ml. of the standard solution to 100 ml. with distilled water. These dilute standards contain respectively 1 μg., 5 μg., 10 μg., and 50 μg. of acetone per ml. Acetone bisulfite equivalents respectively are 2.774 μg., 13.8735 μg., 27.747 μg., and 138.735 μg. per ml.

PROCEDURE

For the estimation of acetone and diacetic acid in normal persons, 10 ml. of urine and at least 10 ml. of protein-free blood filtrate are taken for analysis. This filtrate is obtained by the Folin-Wu procedure (1 volume of blood, 7 volumes of water, and 1 volume of 10 per cent sodium tungstate, and 1 volume of 2/3N sulfuric acid). For pathologic blood or urine containing relatively large quantities of acetone and diacetic acid, less volume may be employed.
The urine or protein-free blood filtrate, after the addition of several glass beads and 3 drops of 1:1 sulfuric acid, is distilled in order to separate the volatile acetone. The all-glass apparatus described by Stotz (21) has been found suitable. The neck of the distilling flask in the area nearer the flask is plugged with a small quantity of glass wool as an additional precaution to prevent bumping, especially when urines are used. The end of the condenser is arranged to dip below the surface of 1 ml. of 2 per cent sodium bisulfite contained in a 15 ml. graduate cylinder or a 15 ml. graduate tube immersed in ice water. The sodium bisulfite solution has been introduced to avoid loss of volatile acetone by the formation of a nonvolatile addition compound. Klein (22) has shown that the bisulfite reaction can be used for the quantitative determination of acetone and diacetic acid in blood filtrates free of sugar. The dissociation of the carbonyl-bisulfite compound can be repressed by using a large excess of sodium bisulfite and lowering the temperature to 5°. Winnick (18) has devised a method for acetone based on the bisulfite-addition compound.

Distillation is carried out with a micro burner at a moderate heat for a period of 2 minutes to yield a total of about 4.5 ml. in the collecting container. The condenser is lifted out of the distillate and about 0.5 ml. is distilled off to rinse the condenser. The surface of the condenser that was dipped into the distillate should also be rinsed and the
washing added to the receiver. The total volume in the receiving tube should be 5 ml.

The contents of the receiving tube are thoroughly mixed, and a 1 ml aliquot (equivalent to 0.20 ml of blood or 2.0 ml urine), is transferred to a graduated test tube. One milliliter of vanillin solution and 2 ml of 40% sodium hydroxide are added. The contents of the tube are thoroughly mixed by gentle shaking, and the tube placed in a water-bath at 60° for exactly 15 minutes.

The mixture is finally diluted with distilled water to 6 ml and read in a spectrophotometer at 415 μm.

**SUMMARY**

Vanillin in an alkaline reaction mixture is a very sensitive reagent for acetone and diacetic acid. A qualitative and a quantitative method have been developed for the acetone bodies in blood and in urine by means of a vanillin-alkali reagent. Acetone and diacetic acid are distilled off and caught in a sodium bisulfite solution, which is allowed to react with vanillin in alkaline medium to form a color complex, which is measured spectrophotometrically.

Vanillin has many advantages over the salicylaldehyde method of Behre. These have been enumerated in the text.

**REFERENCES**