DETERMINATION OF ALCOHOL BY MICRODIFFUSION

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(Received for publication, June 20, 1949)

Numerous methods have been described for the determination of alcohol in small amounts of blood, and an extensive bibliography of such methods has been given by McNally and Coleman (1). Most procedures depend upon the volatility of alcohol and its power to reduce oxidizing agents.

The microdiffusion principle, applied in units designed by Conway (2), can be used to exploit these same properties of alcohol, and enables considerable numbers of determinations to proceed simultaneously with a minimum of attention, so that alcohol determinations can be readily combined with other estimations on the same blood samples.

Winnick (3) has described the application of Conway units to the determination of alcohol in blood, the principle employed being essentially that of evaporation of the blood in the outer compartment and absorption of alcohol in the center compartment by means of dichromate and sulfuric acid, the excess dichromate being finally determined iodometrically. As in the Widmark (4) method, heat must be applied in order to secure rapid absorption, and this in turn necessitates the use of a special fixative. In the present method, saturated potassium carbonate is employed in the outer compartment to assist the expulsion of alcohol, and ready absorption at room temperature is secured by the use of alkaline permanganate in the center chamber. This reagent will absorb free acetone even more readily than alcohol, and in fact the method here described could be used with very slight modification for the determination of free acetone if desired. Free acetone in quantity in the blood is, however, of rare occurrence and the writer has not experienced trouble from this source following experimental administration of alcohol. By a modification mentioned later, alcohol and acetone may be determined in the presence of each other.

Reagents—

1. Approximately 0.04 M potassium permanganate in 3 N potassium hydroxide. 0.63 gm. of potassium permanganate, analytic reagent, is added to 100 ml. of a solution containing 17 gm. of potassium hydroxide, analytic reagent, in distilled water made up to 100 ml. and stored in an amber glass bottle. The bottle is stoppered and shaken at intervals until the permanganate is totally dissolved.

The entry of carbon dioxide to the
alkaline solution should be kept to a minimum during preparation and handling.

2. 10 per cent, weight by volume, barium chloride (BaCl₂·2H₂O) in water. This solution should be kept well stoppered when not in use.

3. A saturated solution of potassium carbonate in water.

4. A 0.01 M solution of thiourea in water. This solution is standardized as described later, and is diluted for immediate use twenty-five times to give 0.0016 M strength. 250 ml. of solution contain 0.76 gm. of thiourea.

5. Commercial water glass, very slightly diluted with water to facilitate application as a lid fixative.

**Procedure**

A number of Pyrex glass Conway units (cleaned as described below), sufficient to include estimations and two blanks, is laid out on the bench and slightly tilted on strips of cardboard 1 to 2 mm. thick. The lids are in position but not smeared with fixative. Before assembly of the units, which are best stored in an inverted position on metal trays in a cupboard, both dish and lid should be inspected to insure the absence of dust, fluff, etc.

0.5 ml. of saturated potassium carbonate is pipetted into the outer compartment of each dish at the lowest level, and the lids are then smeared with fixative and lightly replaced. Into the center compartment of each dish 1 ml. of alkaline permanganate is pipetted carefully by means of a pipette of the Ostwald-Van Slyke pattern which insures very uniform pipetting throughout. The lids are rotated to fix them, and the units covered by a dark cloth until required.

To carry out the estimation, 0.1 ml. of blood is delivered into the outer compartment at the highest level, the lid is firmly sealed, and the dish rotated to mix blood and carbonate and then placed in a dark cupboard. The dish is ready for titration 2 hours later, though the general practice has been to allow 3 hours to insure complete absorption. The dishes may, if desired, be left overnight, the blanks being treated similarly. When blood is obtained from a skin prick, 1 per cent cetyltrimethylammonium bromide may be used for cleansing and disinfecting, with oxalate or fluoride as the anticoagulant. For the estimation of acetone, an absorption period of half an hour was found adequate.

**Titration**—In order to secure the maximum sensitivity and convenience for operations in Conway units, the principle of titrating over the stage of permanganate to manganate was adopted, this first step of reduction being that produced by the alcohol during absorption, so long as the capacity of the permanganate (0.300 to 0.350 mg. of alcohol) was not exceeded. A reducing agent had to be found which would reduce the permanganate
very rapidly to the stage of manganate and very slowly beyond. In order to overcome the difficulty of detecting the end-point in the presence of the intense green color of the manganate, barium chloride was used to precipitate manganate as the insoluble barium salt. Several substances were found to possess the requisite properties, including potassium iodide, potassium thiocyanate, potassium formate, $\alpha,\beta$-unsaturated acids, $\alpha$-hydroxy acids, sodium thiosulfate, and thiourea. Of these, only fumaric acid, sodium thiosulfate, and thiourea were found to react sufficiently rapidly to give sharp end-points. The fumaric acid solution, while satisfactory when fresh, deteriorated very rapidly and was useless after 24 hours. Both thiosulfate and thiourea have been used in practice, but, for reasons given later, thiourea is preferable. Suitable strengths of these solutions were found to be 0.002 $M$ sodium thiosulfate and 0.0016 $M$ thiourea, for use with the standard Conway units of the center compartment capacity to about 4 ml., with a 2 ml. micro burette (with reservoir) graduated in divisions of 0.02 ml., capable of being read by eye to 0.002 ml. Preliminary tests showed that the amount of reducing agent required was proportional to the amount of permanganate present, over the range of the method. Such an experiment may be carried out by diluting the permanganate with 3 $N$ potassium hydroxide. Since every specimen of potassium hydroxide handled by the writer has possessed the power of causing some reduction of permanganate to manganate, it was found necessary, before carrying out dilutions, to add permanganate in small amounts to the potassium hydroxide solution until a faint pink tinge remained, and to make a correction for the small amount of reagent required to reduce the excess. The data presented below indicate that the amount of available oxygen consumed by the alcohol is also proportional to the amount present, over the range of alcohol concentrations to which the method is applicable (0 to 300 mg. per cent with 0.1 ml. samples, 0 to 600 mg. per cent with 0.05 ml.).

In order to perform the titrations 0.2 ml. of 10 per cent barium chloride is added to the center compartment and the contents gently stirred by means of a glass rod drawn down to 1 to 1.5 mm. for a length of 6 cm., and bent, 10 to 15 mm. from the end, to 135°. The stirring is continued during dropwise addition of the reducing agent, till the last trace of pink color has disappeared. Fractional drops may be carried on the rod. The end-point is recognized by the apparent sudden change of color of the precipitated barium manganate viewed through the solution, from steel-blue to dark green.

Calculation of Results—The formula applicable is \((B - E) \times C = \text{mg. of alcohol, or } (B - E) \times 1000C = \text{mg. per cent of alcohol for 0.1 ml. of specimen, where } B = \text{the blank titration in ml., } E = \text{estimation in ml.,}\)
and $C$ = a factor determined experimentally on alcohol solutions of known strength. $C$ will then be the number of mg. of alcohol to which 1 ml. of the reducing agent, e.g. thiourea, is equivalent. The thiourea is best standardized directly against known amounts of alcohol in the Conway units. A suitable standard solution may be made by delivering 1.000 ml. of pure alcohol into a 500 ml. volumetric flask and diluting to the mark with water, or by making a solution of twice this strength and employing a range of dilutions. In the former case, the solution contains 159 mg. per cent of alcohol and 0.1 ml. = 0.159 mg. of alcohol.

As a rigorous test of the method under laboratory conditions, the following experiment was carried out. On 3 separate days, alcohol solutions of fixed strengths were made up and estimated, and on each day fresh dilutions of a stock solution of thiourea were used for titration. The dilute thiourea was ascertained to titrate 1 ml. for $0.177 \pm 0.0015$ mg. of alcohol. The results include all accumulated errors of manipulation arising from dilution of alcohol solutions, dilution of thiourea, measurement and introduction of samples, pipetting of permanganate, and titration error, as well as possible lack of uniformity in cleanliness of dishes, contamination with atmospheric dust, etc.

The results obtained, which are shown in Table I, indicate that, over a considerable range of alcohol concentrations, the method gave good results. It should be stressed that the figures shown are for individual estimations, not the mean of two or more, and it appears that the over-all error, assuming the alcohol dilutions to have been made correctly, was of the order of $\pm 0.003$ mg. for a single estimation. When mean values are calculated, the agreement between experimental and calculated figures is good.

In Table II are given some values obtained on oxalated rat blood to which alcohol was added in known amounts. This method of producing different alcohol concentrations was not completely satisfactory, however, and, as shown in Table III, the results obtained by estimating duplicate blood samples taken from animals to which alcohol had been administered gave close agreement between duplicates. The amount of volatile reducing substance in the blood of untreated animals is also seen to be negligible.

*Notes on Method and Reagents*—The dishes used must be chemically clean. If this is not the case, the fact will be readily detected by the failure of the permanganate to spread at once evenly over the glass surface of the center compartment, and such a dish should be rejected.

The fixative used, water glass, was adopted in preference to fixatives embodying organic substances which might cause reduction of permanganate if a filament is drawn out during removal of the lid for titration. It is easily and completely removed by water and leaves no contaminating
substance on the glass. It should not be allowed to remain in place more than 24 hours, and must be completely removed before acid is used.

**Table I**  
*Determination of Alcohol Solutions*

<table>
<thead>
<tr>
<th>Date</th>
<th>Thiourea used</th>
<th>Alcohol found</th>
<th>Alcohol calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1947 Sept. 11</td>
<td>1.329 ml.</td>
<td>0.235 mg.</td>
<td>0.239 mg.</td>
</tr>
<tr>
<td></td>
<td>0.905 ml.</td>
<td>0.160 mg.</td>
<td>0.159 mg.</td>
</tr>
<tr>
<td></td>
<td>0.607 ml.</td>
<td>0.107 mg.</td>
<td>0.111 mg.</td>
</tr>
<tr>
<td></td>
<td>0.377 ml.</td>
<td>0.067 mg.</td>
<td>0.064 mg.</td>
</tr>
<tr>
<td>Sept. 12</td>
<td>1.360 ml.</td>
<td>0.241 mg.</td>
<td>0.239 mg.</td>
</tr>
<tr>
<td></td>
<td>0.884 ml.</td>
<td>0.156 mg.</td>
<td>0.159 mg.</td>
</tr>
<tr>
<td></td>
<td>0.640 ml.</td>
<td>0.113 mg.</td>
<td>0.111 mg.</td>
</tr>
<tr>
<td></td>
<td>0.320 ml.</td>
<td>0.057 mg.</td>
<td>0.064 mg.</td>
</tr>
<tr>
<td>Sept. 16</td>
<td>1.357 ml.</td>
<td>0.240 mg.</td>
<td>0.239 mg.</td>
</tr>
<tr>
<td></td>
<td>0.901 ml.</td>
<td>0.159 mg.</td>
<td>0.159 mg.</td>
</tr>
<tr>
<td></td>
<td>0.633 ml.</td>
<td>0.112 mg.</td>
<td>0.111 mg.</td>
</tr>
<tr>
<td></td>
<td>0.350 ml.</td>
<td>0.062 mg.</td>
<td>0.064 mg.</td>
</tr>
</tbody>
</table>

Mean calculated from 3 determinations on 4 solutions  
0.239 mg. 0.158 mg. 0.110 mg. 0.062 mg.

1 ml. of thiourea solution = 0.177 ± 0.0015 mg. of ethyl alcohol; error, ±0.003 mg. for a single determination.

**Table II**  
*Recovery of Alcohol Added to Rat Blood in Vitro*

<table>
<thead>
<tr>
<th>Alcohol calculated</th>
<th>Alcohol found</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>1st series</td>
</tr>
<tr>
<td>0.145</td>
<td>0.150</td>
</tr>
<tr>
<td>0.099</td>
<td>0.097</td>
</tr>
<tr>
<td>0.076</td>
<td>0.076</td>
</tr>
<tr>
<td>0.051</td>
<td>0.030</td>
</tr>
</tbody>
</table>

The following routine procedure has proved satisfactory for cleansing the units. As soon as possible after completion of titration, the dishes and lids are thoroughly washed under the tap. The lids may at once be dried with a towel and put away after removal of fluff. The dishes are soaked in dilute hydrochloric acid to remove barium carbonate, then rinsed and filled with dichromate-sulfuric acid cleaning mixture. After standing
1 to 2 hours, they are rinsed with tap water and distilled water, stacked in an inverted position on metal trays, and dried in the oven. They are then stored inverted in a cupboard free from dust until required.

**Table III**

Determ1nations on Blood of Alcohol-Treated and Untreated Animals

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood from rats treated with ethyl alcohol by inhalation; 0.03 ml. specimens from tail measured in specially calibrated capillary pipettes; duplicate determinations</td>
<td>ml.</td>
<td>ml.</td>
<td>ml.</td>
</tr>
<tr>
<td>2.308*</td>
<td>2.300*</td>
<td>2.225*</td>
<td></td>
</tr>
<tr>
<td>2.290*</td>
<td>2.304*</td>
<td>2.252*</td>
<td></td>
</tr>
<tr>
<td>1.912</td>
<td>2.050</td>
<td>1.740</td>
<td></td>
</tr>
<tr>
<td>1.892</td>
<td>2.048</td>
<td>1.763</td>
<td></td>
</tr>
<tr>
<td>1.884</td>
<td>1.982</td>
<td>1.780</td>
<td></td>
</tr>
<tr>
<td>1.900</td>
<td>1.984</td>
<td>1.812</td>
<td></td>
</tr>
<tr>
<td>1.940</td>
<td>1.898</td>
<td>1.930</td>
<td></td>
</tr>
<tr>
<td>1.920</td>
<td>1.920</td>
<td>1.932</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.980</td>
<td>1.812</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.000</td>
<td>1.782</td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood from fasting untreated rabbits; 0.1 ml. specimens from marginal ear vein</td>
<td>ml.</td>
<td>ml.</td>
<td>Reducing substances calculated as acetone (mg. per cent)</td>
</tr>
<tr>
<td>2.580</td>
<td>2.590</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>2.580</td>
<td>2.500</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2.351</td>
<td>2.332</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2.351</td>
<td>2.330</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2.348</td>
<td>2.305</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2.348</td>
<td>2.285</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2.316</td>
<td>2.266</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2.316</td>
<td>2.252</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2.172</td>
<td>2.110</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2.172</td>
<td>2.092</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>1.974</td>
<td>1.977</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 ml. of 0.002 M thiosulfate used = 0.147 mg. of alcohol or 0.078 mg. of acetone.

* Blank.

† Each figure represents blood from a separate animal, the animals being usually bled in pairs.

By treatment of the alkaline permanganate solution with one-fifth of its volume of 10 per cent barium acetate and centrifugation of the barium manganate, a permanganate solution is obtained which is free of manganate and can be titrated under conditions similar to those used in the estimations. Since the available oxygen involved in the reduction of permanganate to manganate is then exactly one-fifth of the total avail-
able oxygen determined iodometrically, it is thus possible to establish
the absolute reducing power of the solutions used for reduction and to
draw conclusions about the oxidation products. Thiosulfate apparently
does not yield a single oxidation product. Calculation of the number of
oxygen equivalents per mole consumed in the oxidation has given the value
7.3 on several occasions, even when freshly prepared thiosulfate solutions
were tested. Since oxidation to sulfate involves uptake of 8 equivalents,
this indicates formation of another oxidation product involving fewer
equivalents per mole. The explanation does not apparently lie in the
preliminary formation of a lower oxidation product, e.g. dithionate, with
slow subsequent oxidation to sulfate, since, if a blank titration is inter-
rupted for half an hour at the half way point and then completed, the end-
point is unchanged. Rather, it appears that the oxidation takes two
courses from the start. An explanation might be found in the possibility
of resonance in the thiosulfate ion, so that the linkage between the sulfur
atoms could behave both as a coordinate and covalent bond, with the
formation of two distinct oxidation products. In the case of thiourea, the
number of equivalents of oxygen consumed per mole was found to be
close to 11, an unexpectedly high figure which suggests cyanate as the final
oxidation product. As thiourea solutions were found to keep well, and,
moreover, since this reagent is relatively insensitive to considerable al-
teration in the titration conditions, such as a gross excess of barium chlo-
ride, thiourea appears the preferable reducing agent.

Comparison of the empirical factor for alcohol with the reducing power
of the solution also permits calculation of the number of equivalents of
oxygen taken up by alcohol during oxidation. This shows that, while the
main product is certainly acetate (with uptake of 4 equivalents per mole
of alcohol), the oxygen consumption is 7 to 9 per cent more than that
calculated for this oxidation, probably the consequence of slight enolization
of the acetaldehyde presumably formed as the intermediate product.
The conditions are thus quite different from those of Friedemann and
Klaas (5), who found oxalate as the product of oxidation of ethyl alcohol
by boiling alkaline permanganate.

The use of potassium carbonate as an agent to increase the vapor tension
of alcohol solutions and so increase absorption in Conway units at room
temperature is referred to in a very brief notice by Ryan et al. (6), appar-
etly in conjunction with Winnick’s (3) absorbent, the statement being
made that absorption was “almost complete” in 1 hour at room tempera-
ture. When the writer was developing the present method some 3 years
ago, it appeared to him that the reaction with dichromate and sulfuric
acid was rather slow for the purpose of a microdiffusion method designed
to operate at room temperature, and this led to the use of alkaline perman-
ganate. Winnick's absorbent has the advantage of not being sensitive to acetone. The acetone-absorbing property of alkaline permanganate has not in the writer's experience proved to be a disadvantage under the conditions of experimental administration of alcohol, and, owing to the rarity of occurrence of free acetone in considerable quantity in the blood, errors due to this substance have been ignored in some methods; e.g., that of Widmark (4).

The method described in this paper can, if necessary, be adapted to the estimation of alcohol in the presence of acetone by substituting for the saturated potassium carbonate solution a buffered solution of potassium sulfite made as follows: 5 gm. of potassium hydroxide are dissolved in 12 ml. of water and cooled, and 11 gm. of potassium metabisulfite are added in small quantities. Finally water is added dropwise until the added salt just dissolves. The pH should then be close to 8. This solution, which keeps for about a week in a stoppered flask, has the property of permitting alcohol to diffuse completely in a 2 hour period, although retaining 93 to 95 per cent of the acetone up to 40 mg. per cent. During the absorption period, a very slight but perceptible reduction of the permanganate in the blank dish takes place (amounting to 0.03 to 0.05 ml. in the titration) due to absorption of sulfur dioxide. The necessity which thus arises of completing the titration within a period of approximately 3 hours from the introduction of the sulfite detracts somewhat from the convenience of the method in this form. Acetone has approximately twice the reducing power of alcohol towards alkaline permanganate. Microdiffusion methods for acetone have been given by Werch (7) and Winnick (8), depending on absorption in the Deniges reagent and bisulfite respectively.

The alkaline permanganate solution deteriorates slowly in the bottle, but, if well protected from the atmosphere, can be kept for 2 to 3 weeks. It should be discarded when the alcohol-oxidizing capacity falls appreciably below 0.300 mg. per ml. (blank titration, 1.7 ml. of 0.0016 M thiourea). When the oxidizing capacity is nearly exhausted by the alcohol present in the unit (titration of residual permanganate <0.15 ml. of 0.0016 M thiourea), formation of oxides of manganese may commence, and this invalidates the calculation based on reduction to manganate. During titration, the removal of manganate ions by barium effectively prevents such further reduction.

Carbon dioxide must not be allowed to gain entry to the potassium hydroxide in large amounts; otherwise this action of the barium is disturbed, but the small amount of carbon dioxide absorbed from the air during titration does not interfere. The precipitated barium manganate is stable in the strongly alkaline solutions employed, but thick crusts of
this substance should not be allowed to accumulate on the stirring rod, as permanganate can be formed on exposure to the air, due to absorption of carbon dioxide.

The alkaline permanganate should not be exposed to an atmosphere contaminated by organic vapors (especially alcohol), dust, or tobacco smoke. The barium chloride also should be protected from alcohol vapor, as appreciable amounts may be absorbed from the air.

Finally it may be mentioned that if alcohol, or better, acetone is introduced into the outer compartment of a Conway unit prepared as described with potassium carbonate as the expelling agent, the change of color of the permanganate solution due to reduction can be easily detected by the eye by comparison with a blank dish after quite a short time. This affords a vivid visual demonstration of the microdiffusion principle in operation.

**SUMMARY**

1. A method of determining ethyl alcohol in blood specimens of 0.1 ml. is described, the principle of microdiffusion being employed.

2. By the use of potassium carbonate as an alcohol-expelling agent and alkaline permanganate for absorption, the Conway units can be conveniently operated at room temperature.

3. A method of titrating alkaline permanganate is described, based on reduction to manganate by thiourea in the presence of barium ions.

4. By the employment of a buffered solution of potassium sulfite to expel alcohol, interference by acetone can, if necessary, be largely eliminated.

The writer desires to express appreciation of the interest shown by Professor F. L. Golla. He is also indebted to Mrs. R. K. Carpenter and Mr. D. Roberts for technical assistance, and to the Monthly Bulletin Research Fund of the British Society for the Study of Addiction, which supported the work.

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