STUDIES IN FAT METABOLISM. I. THE COLORIMETRIC DETERMINATION OF KETONE BODIES IN BIOLOGICAL FLUIDS

George D. Michaels, … , Gerald Liebert, Laurance W. Kinsell

J Clin Invest. 1951;30(12):1483-1485. https://doi.org/10.1172/JCI102557.

STUDIES IN FAT METABOLISM. I. THE COLORIMETRIC DETERMINATION OF KETONE BODIES IN BIOLOGICAL FLUIDS[1]

By GEORGE D. MICHAELS, SHELDON MARGEN,[2] GERALD LIEBERT, AND LAURANCE W. KINSELL

(From the Institute for Metabolic Research of the Highland Alameda County Hospital, Oakland, Cal.)

(Submitted for publication May 18, 1951; accepted August 27, 1951)

The lack of a simple and accurate method for the quantitative determination of ketone bodies in biological fluids accounts in large measure for the relatively small amount of data in the literature relating to ketone metabolism. Most of the methods which have so far been described have required the conversion of β-hydroxybutyric and acetoacetic acids to acetone, the distillation of the acetone so formed, and the quantitative determination of the acetone in the distillate by gravimetric, titrimetric, or colorimetric procedures. The most serious limitation of these procedures lay in the likelihood of loss of acetone during distillation. The necessity for the use of a reflux condenser in the process of conversion of acetoacetic acid and β-hydroxybutyric acid to acetone also constituted a source of possible loss of acetone. The percentage loss, when one was dealing with small quantities of acetone, could be of considerable magnitude.

The method to be described eliminates distillation and refluxing. It makes use of the colorimetric determination of acetone phenylhydrazone described by Greenberg and Lester (1). The following difficulties were encountered in the method as described by these authors:

1. Acetoacetate in the presence of acid dichromate instead of being entirely decarboxylated to acetone is partially and unpredictably converted to compounds other than acetone.

2. As the result of inadequate time of refluxing and possibly to too concentrated a dichromate solution, predictable recovery of β-hydroxybutyrate was not possible.

[1] This work is supported by grants from the Commercial Solvents Corporation and from the Armour Laboratories.

[2] Damon Runyon Clinical Research Fellow, 1949 to present.

EQUIPMENT AND REAGENTS

Equipment

1. Narrow neck, screw top, heavy rim, 4 ounce bottles.[3]
2. Heat resistant, plastic screw caps to fit these bottles.[3]
3. Washers for the screw caps cut from Goodrich Ameripol D rubber sheets.[4] The washers were treated by boiling for 15 minutes in approximately 2 normal sodium hydroxide, washed in running tap water, rinsed in distilled water, soaked in carbon tetrachloride for about 30 minutes, and shaken in the dinitrophenylhydrazine solution for 10 minutes before their initial use. This treatment removed interfering materials from the synthetic rubber. Other rubber products, both natural and synthetic, were tried but were rot satisfactory.

Reagents

1. Barium hydroxide, 0.15 *N*.[5]
2. Zinc sulfate, $ZnSO_4 \cdot 7H_2O$, 2.5 gm./100 cc. of water.[5]
3. Sulfuric acid, 13.6 *N*.
4. Potassium dichromate, 0.4 gm./100 cc. of water.
5. Sodium sulfite, 5.0 gm./100 cc. of water.
6. 2:4 dinitrophenylhydrazine, 0.1 gm. in 100 cc. of 2.0 *N* HCl (filter before use).
7. Carbon tetrachloride (reagent grade).
8. Sodium hydroxide, 10.5 *N*.

DETERMINATION OF KETONES IN WHOLE BLOOD

Precipitation of blood protein—(a modification of the method of Weichselbaum and Somogyi [2])

Add, with shaking, 5 cc. of unclotted blood (collected in a heparin or Wintrobe oxalate tube) to 15 cc. of distilled water in a small flask. Allow five minutes for complete hemolysis.

[3] Owens-Illinois glass bottles No. A-4254 fitted with 20 mm. black plastic screw caps have proven satisfactory.

[4] Acknowledgment is made to the B. F. Goodrich Company for supplies of Ameripol D synthetic rubber.

[5] The concentration of these reagents must be so adjusted that 5.0 cc. of the zinc sulfate solution, diluted to a volume of 25 cc. will require 5.0 cc. of the barium hydroxide solution to produce a permanent pink color, using phenolphthalein as an indicator.

Then add slowly 15 cc. of the barium hydroxide solution (with shaking) followed by 15 cc. of the zinc sulfate solution. Allow at least 10 minutes for complete precipitation of the blood protein. Filter through a good grade paper such as Whatman No. 40.

Hydrolysis and oxidation of keto acids

To each of two bottles, add 5 cc. of the whole blood filtrate and 1 cc. of 13.6 *N* sulfuric acid. Place caps on tightly (with pliers to avoid any possible loss of acetone) and autoclave for 10 minutes at 15 to 20 lbs. pressure. This decarboxylates all of the acetoacetic acid to acetone, and does not affect the β-hydroxybutyric acid.

After removal from the autoclave and cooling to room temperature, add 1 cc. of 0.4% potassium dichromate solution. One bottle is used directly for the determination of preformed acetone plus acetone derived from acetoacetic acid. Oxidation and hydrolysis of β-hydroxybutyric acid is carried out in the other by autoclaving for 30 minutes (bottle tightly sealed). Under these conditions a quantitative conversion of β-hydroxybutyric acid to acetone is achieved.[6]

Quantitative determination of acetone

To each bottle add 1 cc. of 5% sodium sulfite,[7] replace the caps and mix thoroughly. The sodium sulfite reduces the free dichromate which otherwise would oxidize both the hydrazine and the hydrazone. Add 5 cc. of the acid dinitrophenylhydrazine solution and 5.0 cc. of carbon tetrachloride. Recap the bottles and shake for 10 minutes at a rate of approximately 260–275 times per minute (any Kahn shaker is satisfactory). Add 3.0 cc. of 10.5 *N* sodium hydroxide, shake for five minutes, and pour the entire contents of each bottle into a test tube. Remove the supernatant aqueous layer by suction, and compare the color of the carbon tetrachloride fraction with the appropriate standard.

The color of the standards is obtained from the reaction of 5.0 cc. of an aqueous acetone solution (0.1 cc./liter or 7.92 mg./100 cc.); the color of the blanks, from the reaction of 5 cc. of water. In each instance the material is treated in the same manner as the whole blood.

We recommend for acetone concentrations in the blood ranging from 2 to 29 mg./100 cc., a wave length of 420 millimicrons; for a range from 0 to 2 mg./100 cc., a wave length of 375 millimicrons. The use of a Klett-Somerson or comparable colorimeter, employing a blue filter, gives satisfactory results.

Since Beer's Law holds, in a range of 0 to 14 mg./100 cc., the concentration of ketones in the blood (in terms of acetone equivalents, mg./100 cc.) will equal 7.92·Du/Ds (Figure 1).

[6] Standard β-hydroxybutyrate obtained from British Drug Houses, Ltd.

[7] Prepare a fresh solution every week.

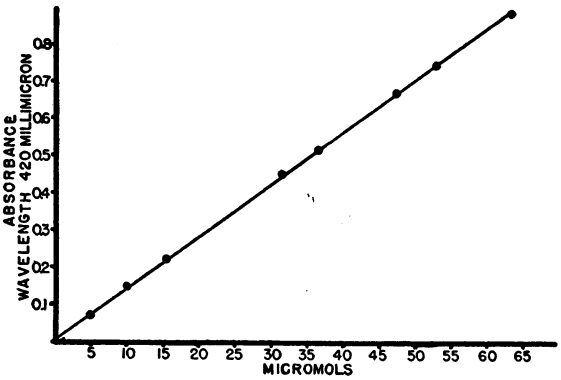

FIG. 1. RECOVERY OF ACETONE

Standard curve.

Procedure for determination of urine ketones

Urine ketones can be determined in the same way. Protein precipitation is unnecessary unless proteinuria is present. A 1:10 dilution has been found to be satisfactory for normal urines.

RESULTS

The recovery of acetoacetic acid and β-hydroxybutyric acid in aqueous solution or added to blood is shown in Table I.

In the present method, in the absence of dichromate, 100% conversion of acetoacetate to ace-

TABLE I

Recoveries of acetoacetic and β-hydroxybutyric acids
(expressed as acetone)

Solvent	Amount added (*mg./100 cc.*)	Amount recovered (*mg./100 cc.*)	Recovery (*per cent*)	
Water	6.34 4.75 3.17 1.56	6.34 4.70 3.20 1.58	100 99 101 101	Acetone in H_2O
	6.34	6.34	100	Acetone in H_2O, treated with $ZnSO_4$ and $Ba(OH)_2$
	4.58	4.53	99	Ethyl acetoacetate from H_2O
	4.58	4.50	98.5	Ethyl acetoacetate from H_2O treated with $ZnSO_4$ and $Ba(OH)_2$
	6.82	6.82	100	β-hydroxybutyric acid from H_2O
	6.82	6.74	98.8	β-hydroxybutyric acid from H_2O treated with $ZnSO_4$ and $Ba(OH)_2$
Blood	0	0.8		Acetoacetic acid
	0	0.25		β-hydroxybutyric acid
	4.58	5.30	98.5	Ethyl acetoacetate
	6.82	6.78	99.4	β-hydroxybutyric acid

All determinations were done in quadruplicate with a variation of less than ± 1%.

TABLE II

Results from analysis of blood of patients

Patient	Acetone and acetoacetic acid (*mg./100 cc.*)	β-hydroxybutyric acid (*mg./100 cc.*)	Total ketones (*mg./100 cc.*)	Diagnosis
D. E. P.	0.31	0.53	0.84	Cirrhosis and nephritis
R. O. M.	0.03	0.35	0.38	Hepatitis, acute
F. E. R.	0.07	0.52	0.59	Hepatitis, chronic
B. R. A.	0.38	0.45	0.83	Jaundice, obstructive
T. U. C.	0.12	0.40	0.52	Cirrhosis
N. I. C.	0.34	0.49	0.83	Congestive failure
L. A. R.	0.33	0.26	0.59	Cirrhosis
S. P. I.	0.20	0.52	0.72	Hypogonadism
R. O. S. 24 hr. fasting	3.5	7.5	11.0	Diabetic
48 hr. fasting	14.5	19.6	34.1	Diabetic
72 hr. fasting	28.5	29.5	58.0	Diabetic

tone is obtained. In the presence of dichromate, as in the method of Greenberg and Lester (1), only a 40-60% recovery results. Recovery of β-hydroxybutyrate by our method was consistently $100 \pm 1\%$. $ZnSO_4$ and $Ba(OH)_2$ did not alter the recoveries.

α-Ketoglutaric acid, lactic acid, and pyruvic acid in concentrations of 1 to 50 mg./100 cc. did not interfere with quantitative recovery of acetone, acetoacetate, or β-hydroxybutyrate.

Blood sugars in excess of 200 mg./100 cc. result in high ketone values. Appropriate dilutions must be made for such bloods.

In Table II are shown the fasting blood ketones obtained in a group of patients, including three consecutive values in a fasting diabetic over a 48 hour period.

SUMMARY

A quantitative colorimetric method for ketones in biological fluids is presented. It eliminates the need for distillation and refluxing.

REFERENCES

1. Greenberg, L. A., and Lester, D., A micromethod for the determination of acetone and ketone bodies. J. Biol. Chem., 1944, **154**, 177.
2. Weichselbaum, T. E., and Somogyi, M., A new method for the determination of small amounts of ketone bodies. J. Biol. Chem., 1941, **140**, 5.