Summary

Six methylol derivatives of nitrofurylvinylogues were prepared from their parent compounds with formaldehyde or paraformaldehyde in water or in organic solvents. Of these, antitumor effect on Ehrlich ascites carcinoma in mice was investigated. 2-Bis(hydroxymethyl)amino-4-[2-(5-nitro-2-furyl) vinyl] pyrimidine (D-ran-methylol) and 2-bis(hydroxymethyl)amino-4-[2-(5-nitro-2-furyl)vinyl]quinoline (2A-4Q-ran-methylol) were revealed to save mice completely from tumor death with the suitable dose intraperitoneally. These two compounds had small toxicities and administrations of 1,000 mg. per kilo did not kill any mouse.

(Received August 6, 1964)

[Chem. Pharm. Bull. 13(5) 534~538 (1965)] *UDC 543.061.544.81:547.473.2*

71. Tsutomu Momose, Yosuke Ohkura, and Kazuya Kohashi: Determination of 3-Hydroxybutyric Acid in Blood *via* Acetone with Trinitrobenzene (Organic Analysis. LX[*1]).

(*Faculty of Pharmaceutical Sciences, Kyushu University*[*2])

The current colorimetric method for the determination of 3-hydroxybutyric acid in blood is based on the oxidation of the acid with a dichromate-sulfuric acid solution. The yielded acetone is distilled[1~3] or extracted[4~6] from the reaction mixture and colored with salicylaldehyde,[1,2] furfural,[3] or 2,4-dinitrophenylhydrazine.[4~6] This method, however, is laborious and usually requires extensive equipment. Therefore it may be unsuitable for routine work in a clinical laboratory.

In the writer's laboratory, a simple piece of oxidation-distillation equipment has been designed which can treat with many samples at the same time. Thus, a simple method of determining 3-hydroxybutyric acid in blood is now presented by combining the procedure with the previously established method of determining acetone and aceto-acetic acid in blood with trinitrobenzene as a color developing agent.[7]

Experimental

Reagents

1,3,5-Trinitrobenzene solution (0.1%), NaOH solution (1.8%), NaH_2PO_4 solution (18 g./dl.), Na_2WO_4 solution (7.5 g./dl.) and $KAl(SO_4)_2$ solution (7.2 g./dl.) are prepared in the same way as described in the previous paper.[7]

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*1 Part LIX: Bunseki Kagaku, **14**, 240 (1965).

*2 Katakasu, Fukuoka (百瀬 勉, 大倉洋甫, 小橋一彌).

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2) J.Z. Rutman: J. Lab. Clin. Med., **41**, 648 (1953); O. Hansen: Scand. J. Clin. & Lab. Invest., **11**, 259 (1959).
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4) L.A. Greenberg, D. Lester: J. Biol. Chem., **154**, 177 (1944).
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7) T. Momose, Y. Ohkura, K. Kohashi, R. Nagata: This Bulletin, **11**, 973 (1963).

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Sulfuric acid solution : Prepare 0.4*N* solution.
Potassium dichromate-sulfuric acid solution : Dissolve 350 mg. of $K_2Cr_2O_7$ in 100 ml. of 15*N* sulfuric acid.
Calcium zinc *dl*-3-hydroxybutyrate : Prepare according to the Shaffer-Marriott method,[8] and recrystallize from dil. EtOH to colorless needles, m.p. 241~243° (decomp.).

Equipment

Oxidation-distillation tube and acceptor : Make this tube and acceptor of hard glass in the shape and size as shown in Fig. 1, and mark 2.0 and 5.0 ml. levels on the acceptor.

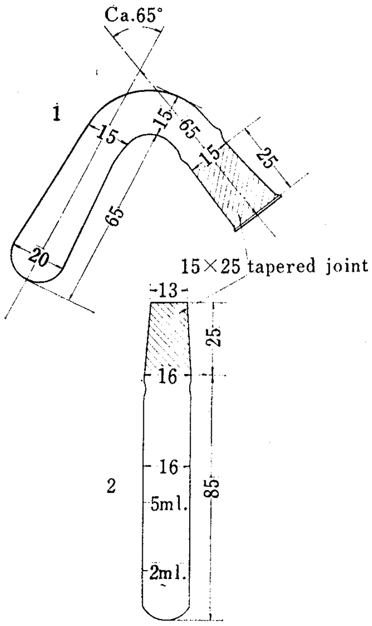

Fig. 1. Oxidation-Distillation Tube (1) and Acceptor (2) (Unit : mm.)

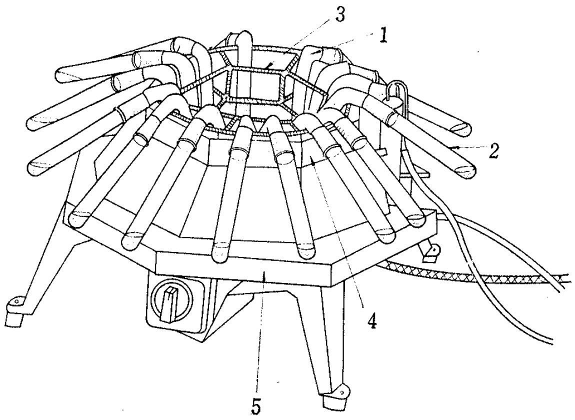

Fig. 2. Oxidation-Distillation Unit.

1 : oxidation-distillation tube
2 : acceptor
3 : rack
4 : water bath
5 : electric heater

Procedure

Dilute 0.20 ml. of blood with 1.80 ml. of H_2O in a centrifuge tube, add 1.00 ml. of Na_2WO_4 solution, and mix. Add 1.00 ml. of $KAl(SO_4)_2$ solution to the mixture, and mix well. Cover the tube with a piece of Parafilm, and centrifuge the mixture.

Pipette 2.00 ml. of the resulting supernatant clear solution into an oxidation-distillation tube, add 0.50 ml. of H_2SO_4 solution, and weigh the tube. Pack the tube in a heating basket,[9] and heat in a boiling water bath for 30 min. to remove the preformed acetone and acetoacetic acid from the deproteinized blood solution into the air and then cool in running water. Add sufficient amount of H_2O to the tube to make the original weight. Add 0.50 ml. of $K_2Cr_2O_7$-H_2SO_4 solution, and mix. Place 1.00 ml. of H_2O into the acceptor as trapping water. Join firmly the oxidation-distillation tube with the acceptor, seal with a strip of adhesive tape, put in a boiling water bath in the same way as shown in Fig. 2, and heat for 90 min. to distill the acetone formed during the oxidation, keeping the temperature of the acceptor at 25~30°. After cooling in room temperature, carefully remove the oxidation-distillation tube from the acceptor to avoid contaminating the trapped acetone solution with the residuum of the oxidation mixture.

Add H_2O to the 2.0 ml. mark on the acceptor, and add successively 1.00 ml. of trinitrobenzene solution and 1.00 ml. of NaOH solution. At the same time, prepare a reagent blank by adding the color developing agents to 2.00 ml. of H_2O in another acceptor in the same way. Dip the acceptors in a water bath of 37° for 1 hr. to develop the color under protection from light. After cooling in an ice bath, add NaH_2PO_4 solution to the 5.0 ml. mark on the acceptors, heat again in a boiling water bath for 15 min., and cool in running water. Measure the absorbance of sample at 480 mμ with the reagent blank, and read the value of 3-hydroxybutyric acid on the calibration curve.

Calibration Curve

Dissolve 55.8 mg. of Ca Zn 3-hydroxybutyrate in H_2O and make up to 500 ml. This solution contains 50 μg./ml. of 3-hydroxybutyric acid as acetone. Using this solution, prepare various diluted solutions which correspond to 2.5, 5, 10, 15, and 20 μg./ml. of 3-hydroxybutyric acid as acetone.

8) P. A. Shaffer, W. M. Marriott : J. Biol. Chem., **16**, 265 (1913).
9) T. Momose, A. Inaba, Y. Mukai, M. Watanabe : Talanta, **4**, 33 (1960).

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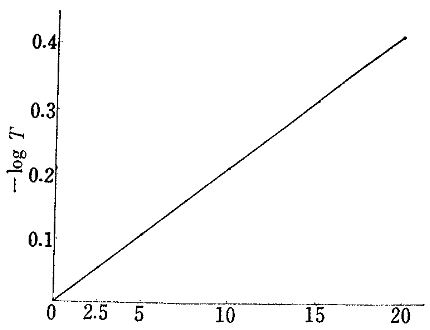

Fig. 3. Calibration Curve

mg./dl.: in Blood
μg./ml.: 3-Hydroxybutyric acid as acetone

Pipette three aliquots of 2.00 ml. of each solution into centrifuge tubes, and treat in the same way as for a blood sample. Prepare three aliquots of the reagent blank as mentioned before. Measure the absorbance of each tube with the reagent blank, which is prepared by mixing three aliquots.

The calibration curve thus drawn up is shown in Fig. 3.

The number of μg./ml. as acetone in diluted 3-hydroxybutyric acid corresponds to the value of the acid in blood calculated in mg./dl. as acetone.

Results and Discussion

A preliminary study was first carried out on the recovery of acetone by distilling aqueous acetone solutions to find out optimum conditions in the distillation procedure. The shapes and sizes of the oxidation-distillation tube and the acceptor affected the distillation ratio of acetone and the equipment in Fig. 1 proved to give satisfactory results. The heating time for distillation also affected the ratio, and the selected time of 90 minutes, gave the maximum recovery of 95±2%. It was of interest to note that the temperature of the acceptor played an important role for the distillation of acetone in a closed tube as demonstrated by Lyon and Bloom.[3] The recovery of acetone was studied by distilling a known amount of acetone with the proposed equipment, maintaining the temperature of the acceptor at 5, 10, 20, 25, 30, and 35°. Results are shown in Table I. A temperature below 20° gave apparently a lower recovery, and therefore, the acceptor should be kept at 25~30° to gain the maximum recovery.

Table I. Effect of the Temperature of Acceptor on the Recovery of Acetone

Temperature of acceptor (°C)	Acetone tested (μg./ml.)	Acetone found (μg./ml.)					Mean recovery (%)
5	10.0	4.32	3.36	5.15	3.85	4.92	43
10	10.0	9.25	9.35	9.30	9.30	8.10	91
20	10.0	9.25	9.40	9.36	9.36	9.69	94
25	10.0	9.75	9.55	9.75	9.55	9.47	96
30	10.0	9.55	9.75	9.93	9.90	9.45	97
35	10.0	9.53	9.30	9.55	9.36	9.40	94

2.00 ml. of acetone solution and 1.00 ml. of water were distilled into 1.00 ml. of water, diluted to 2.00 ml. with water, and colored with trinitrobenzene by the procedure.

The velocity of oxidation of 3-hydroxybutyric acid to acetone depended on the concentrations of potassium dichromate and sulfuric acid.[10] The concentrations described under Reagents were selected to obtain the maximum yield over a wide range of concentrations of 3-hydroxybutyric acid from 2.5 to 20 μg./ml. as acetone. The yield of acetone increased rapidly with increased heating time for the first 60 minutes, and reached its maximum, 94%, in 90 minutes.

3-Hydroxybutyric acid or its sodium salt had been used to prepare the standard solutions. These reagents, however, were hygroscopic and might be unsuitable for the purpose. In this study, calcium zinc double salt of the acid was used because of the convenience of its preparation and storage.

The blood solution might be deproteinized by barium hydroxide and zinc sulfate or by the mixture of sodium hydroxide and zinc sulfate, but sodium tungstate and alum

10) J. Procos: Clin. Chem., **7**, 97 (1961).

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were chosen in this study as well as in the determination of acetone and acetoacetic acid in blood[7] because of the convenience of their preparation, storage, and use. The optimum concentrations of both reagents were those described under Reagents.

The removal of acetone and acetoacetic acid which occurred originally in blood was performed by heating the deproteinized blood solution in a boiling water bath with sulfuric acid. The results depended on the heating time and the concentration of sulfuric acid, and the selected time, 30 minutes, and the concentration, 0.4*N*, were preferable. The lost water in the removal procedure was supplied by weighing the oxidation-distillation tube to restrict the concentrations of potassium dichromate and sulfuric acid for the oxidation of 3-hydroxybutyric acid as described above.

Recovery tests of the present method of determination were carried out by adding known amounts of 3-hydroxybutyric acid to blood solutions. The results are shown in Table II, which shows an average recovery of 97.5%.

Table II. Recovery Test of 3-Hydroxybutyric Acid
(Mean value of 4 determinations)

Blood No.	Initial blood 3-hydroxybutyric acid value (mg./dl. as acetone)	3-Hydroxybutyric acid added (mg./dl. as acetone)	Total 3-hydroxybutyric acid value found (mg./dl. as acetone)	3-Hydroxybutyric acid recovered (%)
1	0.85	1	1.80	97
2	0.52	1	1.46	96
3	0.45	1	1.40	97
4	0.85	3	3.75	97
5	0.52	3	3.45	98
6	0.45	3	3.38	98
7	1.90	10	11.90	100
8	1.15	10	11.00	96
9	2.38	10	12.25	98
			mean	97.5%

Some substances which might occur in blood, including several active methylene compounds, gave practically no interference on the method in a normal range of their concentrations in blood. Those were acetone, acetoacetic acid, acetaldehyde, pyruvic acid, 2-oxoglutaric acid, creatinine, creatine, ascorbic acid, and lactic acid. Glucose gave a weak influence on the value, but it can be corrected by substracting 0.25 mg./dl. of 3-hydroxybutyric acid as acetone per 100 mg./dl. of glucose.

Some anticoagulants which might be added in blood gave no influence on the value of 3-hydroxybutyric acid.

Table III. Parallel Test of 3-Hydroxybutyric Acid with a 2,4-Dinitrophenylhydrazine Method
(Mean value (mg./dl. as acetone) of 4 determinations)

Blood No.	Present[a] method	2,4-Dinitrophenylhydrazine method	Blood No.	Present[a] method	2,4-Dinitrophenylhydrazine method
1	2.35	2.60	6	1.25	1.30
2	0.40	0.36	7	2.76	2.80
3	1.42	1.38	8	1.01	1.00
4	2.85	2.90	9	2.81	2.77
5	0.25	0.31	10	3.50	3.62

a) Corrected by substructing the erroneous value caused by glucose.

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Those tested were EDTA, sodium fluoride, potassium oxalate, and ammonium oxalate. Sodium citrate gave a larger value in the method, which might form acetone by the oxidizing agent, and hence it should not be used in this method.

The results of parallel tests with a 2,4-dinitrophenylhydrazine method[5] on blood are shown in Table III. The individual value in both methods was in good agreement in an experimental error. The precision of this method was examined by carrying out 25 separate analysis on blood, and the standard deviation was 4.1% for a mean value of 2.89 mg./dl. of 3-hydroxybutyric acid as asetone. This method is accurate enough as a distillation method and may be acceptable for routine work.

Summary

A simple method has been presented for the determination of 3-hydroxybutyric acid in blood. It is based on the oxidation of 3-hydroxybutyric acid to acetone with potassium dichromate in sulfuric acid, which is distilled during the oxidation and determined by the previously established method with trinitrobenzene as a color developing agent.

Simple and compact oxidation-distillation equipment has been designed and successfully used, which made possible to analyze many samples at the same time.

(Received December 1, 1964)

[Chem. Pharm. Bull. 13(5) 538~545 (1965)]

UDC 547.94 : 582.675.4

72. Masao Tomita, Toshiro Ibuka,*1 Yasuo Inubushi,*2 Yasuo Watanabe, and Matao Matsui*3 : Studies on the Alkaloids of Menispermaceuos Plants. CCX.*4 Alkaloids of *Stephania japonica* MIERS.(Suppl. 9).*5 Structure of Hasubanonine and Homostephanoline.*6

(*Faculty of Pharmaceutical Sciences, Kyoto University,*1 Faculty of Pharmaceutical Sciences, Osaka University*2 and Dai-ichi College of Pharmaceutical Sciences*3*)

Hasubanonine, m.p. 116°, $C_{21}H_{27}O_5N$, was first isolated and named by Kondo, *et al.*[1] in 1951 from *Stephania japonica* MIERS. The alkaloid contains four methoxyl groups, one N-methyl group and one conjugated carbonyl group. Hasubanonine gave hemipinic acid on permanganate oxidation and phenanthrene on zinc dust distillation.[1] Hofmann degradation of its methiodide afforded a methine base,[1,4] $C_{21}H_{27}O_5N$, which on heating with acetic anhydride generated 2-dimethylaminoethanol and acetylhasubanol (monoacetoxy-trimethoxyphenanthrene) (Ia). Hydrolysis of Ia followed by methylation gave

*1 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto (富田真雄, 井深俊郎).
*2 Toneyama, Toyonaka, Osaka (犬伏康夫).
*3 Takamiya-tamagawa-cho, Fukuoka (渡辺恭男, 松井又夫).
*4 Part CCIX. M. Tomita, Y. Okamoto : Yakugaku Zasshi, **85**, 456 (1965).
*5 (Suppl. 8). Y. Watanabe, H. Matsumura : *Ibid.*, **83**, 991 (1963).
*6 A preliminary communication of this work appeared in Tetrahedron Letters, No. 40, 2937 (1964).
1) H. Kondo, M. Satomi, T. Odera : Ann. Rep. ITSUU Lab., **2**, 35 (1951).
2) M. Satomi : *Ibid.*, **3**, 37 (1952).

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