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Studies on the Voges-Proskauer Reaction. IX.[1,2] A New Spectrophotometric Determination Method for Moroxydine by Voges-Proskauer Reaction

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A simple and specific method for the determination of moroxydine in serum was established by the use of ion-exchange chromatography and a modified Voges-Proskauer colorimetric reaction. Moroxydine over the range of 5 to 70 μg was determined by the measurement of absorbance at the absorption maximum (520 nm) of the colored product formed in the reaction of moroxydine with diacetyl-3,5-dihydroxybenzoic acid reagent in alkaline solution.

An anion-exchange column chromatography (carboxylate resin) was effectively used to remove the interferences present in the serum. The rabbit serum containing moroxydine was applied directly to the column, without pretreatment, and the column was eluted first with 0.01 M NaOH, followed by 0.2 M NaOH. The recovery of moroxydine added to serum was between 99 and 102% and the coefficient of variation was between 1.49 and 2.27%.

Keywords——colorimetry; determination of moroxydine; antiviral agent; modification of Voges-Proskauer reaction with diacetyl-3,5-dihydroxybenzoic acid reagent; separation of moroxydine in rabbit serum by ion-exchange column chromatography

A few methods for the determination of moroxydine (N′,N′-anhydrobis(β-hydroxyethyl)biguanide, ABOB), an antiviral agent, have been reported.[4–7] In biological fluids, moroxydine is determined by the colorimetric method of Andes and Myers[6] using potassium ferricyanide and sodium nitroprusside in alkaline medium, or by a differential spectrophotometric method of Flanagan, *et al.*[7] using bromine water. However, these methods are not satisfactory in sensitivity, specificity, and simplicity of the procedure.

We have developed a simple, specific procedure for the determination of moroxydine in serum. This method is based on a modified Voges-Proskauer colorimetric reaction using diacetyl and 3,5-dihydroxybenzoic acid. The present report describes the colorimetric conditions for the analysis of moroxydine and the application of this method to the determination of moroxydine in serum by an ion-exchange chromatography.

Experimental

Materials——Creatine monohydrate, arginine, methylguanidine hydrochloride, and guanidine hydrochloride were purchased from Sigma Chem. Co., U.S.A. Moroxydine hydrochloride was obtained from Wakamoto Pharmaceutical Co., Tokyo. Diacetyl and other chemicals were of reagent grade, and 3,5-dihydroxybenzoic acid was purified by recrystallization from H_2O. For chromatography, a column (5 × 0.7 cm) of weakly acidic, carboxylic acid ion-exchange resin (Amberlite CG-50, Type I, 100—200 mesh; Organo Co., Tokyo) was used. The resin was washed with 25 ml of 1 M NaOH solution. Before use, the resin was washed with 25 ml of distilled water and equilibrated with 25 ml of 0.01 M NaOH solution.

1) Part VIII: T. Sakaguchi and S. Tanabe, *Yakugaku Zasshi*, **97**, 223 (1977).
2) This paper constitutes Part VII of Reaction of Guanidines with α-Diketones by S. Tanabe and co-workers. Part VI: S. Tanabe and T. Sakaguchi, *Chem. Pharm. Bull.* (Tokyo), **26**, 423 (1978).
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6) J.E. Andes and V.C. Myers, *J. Lab. Clin. Med.*, **22**, 1147 (1937).
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Reagents——(A) Aqueous stock solution of 1% diacetyl. (B) Aqueous solution of 3% 3,5-dihydroxybenzoic acid-0.025% diacetyl; The reagent was prepared freshly by dissolving 3 g of 3,5-dihydroxybenzoic acid and 2.5 ml of 1% diacetyl stock solution in 100 ml of distilled water. (C) 5 M, 1 M, 0.2 M, and 0.01 M NaOH. Standard solution of moroxydine: 3.5 mg/dl/250 μg/dl as the concentration of moroxydine. Preparation of the moroxydine added rabbit serum: To 2.8 ml of rabbit serum, 0.2 ml of moroxydine hydrochloride solution of various concentrations (50, 25, and 12.5 mg/dl as the concentration of moroxydine) was added.

Procedure——Standard Method: To 2 ml of aqueous solution of moroxydine hydrochloride placed in a test tube, 1 ml of 3% 3,5-dihydroxybenzoic acid–0.025% diacetyl solution is added first, and then 2 ml of 5 M NaOH. After mixing well, the tube is immersed in a water bath of 40° for 30 min. The tube is then removed from the water bath and cooled in running water. The absorbance is measured within 20 min at 520 nm, using a reagent blank as a reference.

Determination of Moroxydine in Rabbit Serum: One milliliter of moroxydine-added rabbit serum is applied directly to the column, without pretreatment. The column is eluted first with 14 ml of 0.01 M NaOH, followed by 15 ml of 0.2 M NaOH. The effluent from 0.01 M NaOH and the first 2-ml effluent from 0.2 M NaOH are discarded, and the next 4 ml, which contains moroxydine, is collected into a 10-ml graduated cylinder. Two milliliters of the effluent is assayed as described above.

Results and Discussion

1) Colorimetric Conditions for the Analysis of Moroxydine Absorption Spectrum

Moroxydine reacts with diacetyl and 3,5-dihydroxybenzoic acid in alkaline solution to give a reddish orange compound which exhibits a maximum absorption at 520 nm.

Effect of Reagent Concentration——The effect of the concentration of diacetyl, 3,5-dihydroxybenzoic acid, and NaOH on color intensity was examined with 2 ml of sample solution containing 37.9 μg of moroxydine chloride. The absorbance was measured after standing for 60 min at room temperature. The maximum absorbance was obtained when the concentration of diacetyl, 3,5-dihydroxybenzoic acid, and NaOH was more than 0.025%, 2%, and 5 M, respectively. Taking into consideration the color developed in the blank, 0.025% diacetyl, 3% 3,5-dihydroxybenzoic acid, and 5 M NaOH were employed in the standard procedure.

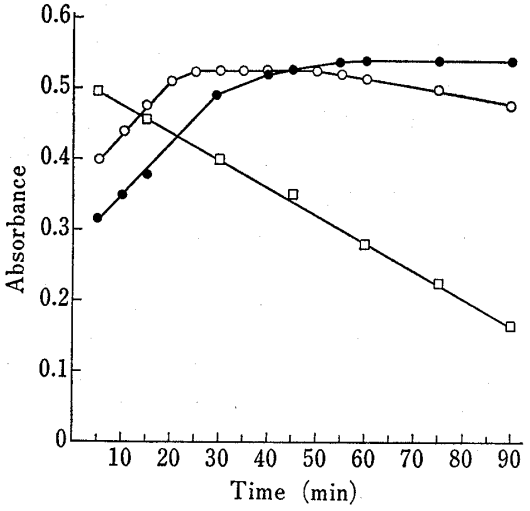

Fig. 1. Effect of Reaction Time and Reaction Temperature

●—●: 30°, ○—○: 40°, □—□: 60°.

Effect of Reaction Time and Temperature——The effect of the reaction time and temperature on the color intensity is shown in Fig. 1. The reaction time required for the optimum color development was about 60 min at 30° and 25 to 45 min at 40°. When the reaction was carried out at 60°, the absorbance reached the maximum immediately, then faded rappidly. The measurement absorbance was carried out within 20 min after reacting for 30 min at 40°.

Working Curve for Moroxydine——Moroxydine over the range of 5—70 μg (final concentration, 1—14 μg/ml) was determined by this method. On the determination of moroxydine, this modified Voges-Proskauer reaction ($\varepsilon' = 1.27 \times 10^4$) was about ten times more sensitive than a general Voges-Proskauer reaction ($\varepsilon' = 1.57 \times 10^3$) with diacetyl and 1-naphthol.[8]

2) Determination of Moroxydine in Serum

The modified Voges-Proskauer reaction is specific for guanidines involving biguanides. Therefore, this reaction was applied to the determination of moroxydine in rabbit serum.

8) T. Nishimura, S. Tanabe, H. Toku, T. Kono, Y. Sakabe, and T. Sakaguchi, *Chem. Pharm. Bull.* (Tokyo), 17, 639 (1969).

In biological fluids, a large number of Voges-Proskauer positive compounds, such as guanidino acids and alkylguanidines, are present. Ion-exchange chromatography (a weakly acidic ion-exchange resin) gave a good result for the isolation of moroxydine from these interferences. Fig. 2 illustrates a typical chromatographic pattern of a synthetic mixture of arginine, creatine, moroxydine, methylguanidine, and guanidine. Moroxydine was separated distinctly from other guanidines in the fraction Nos. 14—17 (the effluent was collected in 1-ml fractions with an automatic fraction collector) with 0.2 M NaOH as eluant. A typical chromatographic pattern obtained with rabbit serum is also shown in Fig. 2. When 1 ml of serum was applied

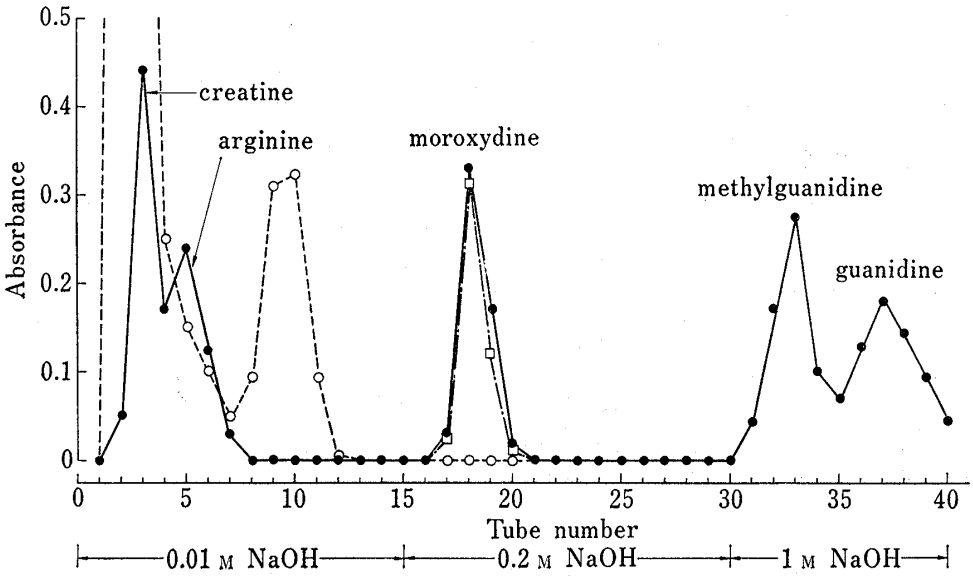

Fig. 2. Chromatogram of Moroxydine, Cuanidines, and Rabbit Serum

●——●: mixture of synthetic moroxydine and guanidines,
○------○: rabbit serum, □—·—·—□: moroxydine added to rabbit serum.

directly to the column, without pretreatment, Voges-Proskauer positive fractions containing protein and guanidines did not interfere with analysis of moroxydine because they were eluted by 0.01 M NaOH. The recovery of known amount of moroxydine added from rabbit serum was between 99 and 102% and the coefficient of variation was between 1.49 and 2.27%, as shown in Table I. Moroxydine in serum over the range of 10 to 70 μg was determined by the column chromatographic method.

Table I. Recovery of Moroxydine from Rabbit Serum

Added (μg)	50.0	25.0	12.5
Found (μg)	51.2	25.4	13.1
	49.6	24.9	12.8
	49.6	24.9	12.7
	48.9	24.5	12.4
Mean (μg)	49.8	24.9	12.8
±SD	0.97	0.37	0.29
CV (%)	1.95	1.49	2.27
Recovery (%)	99.6	99.6	102.4

SD: standard deviation, CV: coefficient of variation.

The developed method can be used for the measurement of moroxydine concentration in serum after the administration of therapeutic doses to human, and the technique is simple and rapid compared with that of the method of Flanagan, *et al.*[7]

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