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Effect of Acetohexamide (a Sulfonylurea Hypoglycemic Agent) in Blood Plasma on Creatinine Assay in Clinical Laboratory Tests[1)]

SADAO BABA, TOMOKO BABA,[2a)] and TAKAO IWANAGA[2b,c)]

Central Clinical Laboratories, Tohoku University School of Medicine[2a)] and Central Clinical Laboratories, Shinshu University Hospital[2b,c)]

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A study has been made of the effect of acetohexamide [N-(*p*-acetylphenylsulfonyl)-N′-cyclohexylurea] in blood plasma on the creatinine assay procedure based on the Jaffe reaction. An analytical method has been proposed in order to determine the drug in plasma and to clarify the causative relationship between plasma acetohexamide and creatinine values. The method involves the formation of 2,4-dinitrophenyl (DNP) derivative of the liberated cyclohexylamine from the drug on hydrolysis and its determination by high-pressure liquid chromatography with an ultraviolet detection.

It was found that false positive errors in creatinine assay values due to an oral dose of 500 mg of the drug ranged from about 40 to 100 percent in the given subjects with diabetes.

The change with time in the concentration of plasma glucose was also described.

Keywords——sulfonylureas; acetohexamide; diabetes mellitus; drug interference; clinical laboratory tests; plasma creatinine determination; Jaffe reaction; 2,4-dinitrophenyl derivative; high-pressure liquid chromatography; plasma glucose estimation

Interference of drugs with clinical laboratory test values has become of major interest. Various medications affecting the clinical laboratory test results have appeared in the literature.[3–5)] In our laboratories, some hundred drugs commonly in use were examined *in vitro* for their possible effect on the creatinine assay procedure based on the Jaffe reaction.[6,7)] Of the drugs studied, acetohexamide [N-(*p*-acetylphenylsulfonyl)-N′-cyclohexylurea], an oral antidiabetic agent belonging to sulfonylurea series, was found to give false positive errors in the determination of plasma creatinine values. During recent years many methods have been presented for the estimation of sulfonylurea derivatives. These methods are based primarily on gas chromatography[8–10)] and polarography[11)] or colorimetry.[12–14)] In the present paper, a high-pressure liquid chromatographic method of estimating plasma acetohexamide was developed to discuss its *in vivo* interference with the Jaffe reaction for the determination of plasma creatinine.

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2) Location: *a*) *Seiryo-cho, Sendai 980, Japan*; *b*) *Asahi, Matsumoto 390, Japan*; *c*) Present address: *Kashiwagi 2-1-6, Sendai 980, Japan.*
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Experimental

Materials——2,4-Dinitrofluorobenzene (DNFB) was of reagent grade. DNFB reagent (0.1%) was prepared by dissolving DNFB in isoamyl acetate.

Drug Standard: Acetohexamide was obtained as crystalline powder from Shionogi and Co., Ltd. (Osaka, Japan) and once recrystallized from 80% ethanol [colorless needles, mp 181—182° (uncorr.)]. The stock solution was prepared by dissolving 200 mg of acetohexamide in 100 ml of 0.02 N NaOH and was added to plasma for the determination of recovery rates. The standard solution was prepared by diluting 10 ml of the stock solution to 100 ml with water.

Internal Standard: *n*-Hexylamine was used as internal standard. A stock solution was prepared by placing 0.1 ml of *n*-hexylamine and a few drops of *β*-thiodiglycol in a 10-ml volumetric flask and diluted with isoamyl acetate. The working internal standard was then prepared by pipetting 1 ml of the stock solution and by diluting to 250 ml with isoamyl acetate.

2,4-Dinitrophenyl Derivatives of Amines: Authentic DNP-derivatives of cyclohexylamine and *n*-hexylamine were as in the preceding paper.[15]

All other chemicals used were of the reagent grade from commercial sources unless otherwise stated.

High-Pressure Liquid Chromatography——The apparatus used was a Jasco Model FLC-100 high-pressure liquid chromatograph (Japan Spectroscopic Co., Ltd., Tokyo, Japan) equipped with a reversed-phase column (octadecylsilane, 50 cm × 2.3 mm i.d.) and a variable wavelength ultraviolet spectrophotometer (Jasco Model UVIDEC-100) monitoring the column effluent at 350 nm. The mobile phase was methanol–water (65: 35) and its flow rate was 0.47 ml per min.

Subject and Dosing——Blood samples were obtained from 5 diabetic outpatients on medical treatment. Acetohexamide was orally administered to each subject in a dose of 500 mg (Dimelin tablet, Shionogi and Co., Ltd.,) before breakfast. Under the physician's care, each subject was fasted for another 5 hours after dosing. The samples were withdrawn immediately before and at every one hour after the administration, and were transferred into the test tubes containing a small amount of sodium fluoride.

Determination of Creatinine and Glucose in Plasma——The method used for the determination of plasma creatinine was essentially based on the Jaffe reaction.[6,7] One ml of plasma was diluted with 8 ml of water. To the solution, 0.5 ml of 2/3 N H_2SO_4 and 0.5 ml of 10% sodium tungstate (dihydrate) were added and stirred thoroughly. The mixture was allowed to stand for 5 min and filtered through a filter paper. To 2.5 ml of the filtrate or 2.5 ml of the standard solution of creatinine (consisting of 0.5 ml of a 1 mg/100 ml solution and 2 ml of water), was added 1 ml of 0.04 M picric acid followed by 1 ml of 0.75 N NaOH. Each solution was incubated at 37° for 15 min and, after standing at room temperature for an additional 15 min, the absorbance was read at 515 nm against the reagent blank. According to the manufacturers' instructions, concentration of plasma glucose was estimated with the Glucose Kit-N (Nippon Shoji Kaisha Ltd., Osaka, Japan) containing *o*-toluidine, boric acid and acetic acid.

Determination of Plasma Concentration of Acetohexamide——The preparation of 2,4-dinitrophenyl derivative of cyclohexylamine[16] was carried out with suitable modification of Spingler's method.[12] To 0.5 ml of plasma in a glass-stoppered tube, 2 ml of 0.02 N HCl and 5 ml of isoamyl acetate were added. The mixture was shaken vigorously for 2 min and centrifuged. After centrifugation, 0.5 ml of internal standard solution and 0.5 ml of 0.1% DNFB reagent were added to 3 ml of the organic layer. The solution was heated at 120±3° for 10 min in a glycerol bath and was then allowed to stand at room temperature. An aliquot (5 μl) of the solution was directly injected with a syringe into a chromatograph.

Results and Discussion

The chromatograms obtained from the drug standard, the drug-free plasma and the plasma containing the drug are illustrated in Fig. 1 (a, b, c).

A small portion (0.025—0.5 ml) of the standard solution of acetohexamide was pipetted in amounts corresponding to 1 mg to 20 mg/100 ml concentrations. The serial solutions were made each to 0.5 ml with water and were treated in the same manner as described for plasma samples. The calibration curve was constructed by plotting the peak height ratio of aceto-

15) S. Baba, I. Hashimoto, and Y. Ishitoya, *J. Chromatogr.*, **88**, 373 (1974).

16) The compound reported as 2,4-dinitrophenyl derivative of intact acetohexamide in a previous paper[15] was proved to be that of cyclohexylamine (a moiety of hydrolysates of acetohexamide as mentioned above) after reinvestigation by elemental analysis, mixed melting point determination and chromatographic behaviors. The decomposition process of sulfonylureas has been already described in the literature.[12,13,17]

17) A. Häußler and P. Hajdú, *Arch. Pharm.*, **295**, 471 (1962).

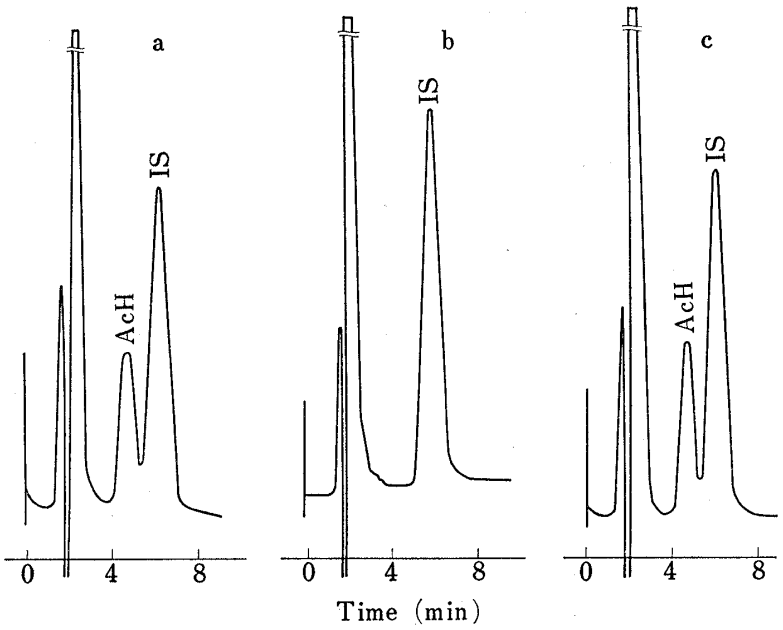

Fig. 1. Typical High-Pressure Liquid Chromatograms

a. the drug standard. b. the drug-free plasma from a patient. c. the plasma from a patient 2 hours after receiving 500 mg of the drug. AcH, acetohexamide (DNP-cyclohexylamine); IS, internal standard (DNP–*n*-hexylamine).

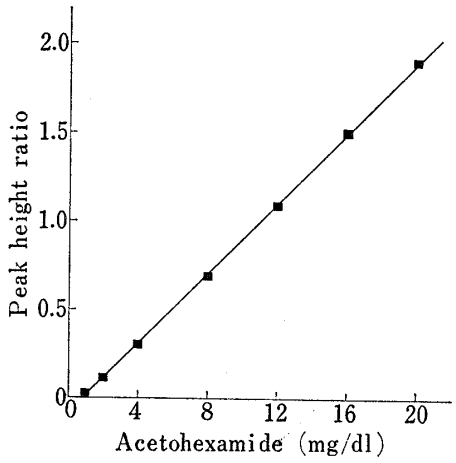

Fig. 2. Calibration Curve for Acetohexamide

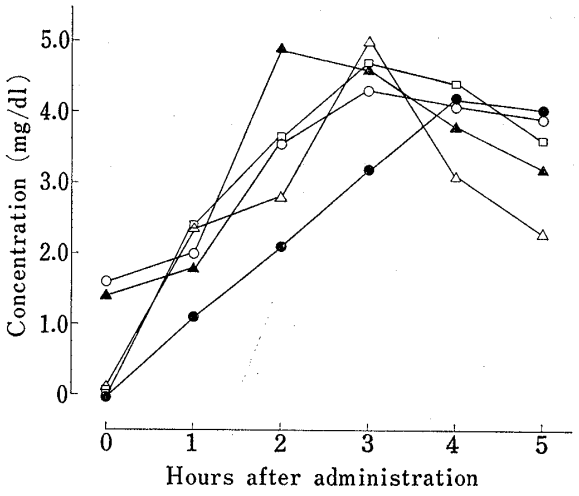

Fig. 3. Changes with Time in Concentration of Total Acetohexamide in Plasma

Fourteen hours before this administration, two subjects (○—○ and ▲—▲) were orally given in a dose of 500 mg.

hexamide to that of the internal standard. As shown in Fig. 2, a straight line with small values of *y*-intercept was obtained in the concentration ranges 1—20 mg/100 ml. The lower limit of the determination was 50 ng per 5 μl injection.

The recovery of the drug after adding known amounts to the drug-free plasma is listed in Table I. The recovery is adequate.

As described in the literature,[18–20] the major metabolite of acetohexamide was L-(−)-hydroxyhexamide [N-(*p*-hydroxyethylphenylsulfonyl)-N′-cyclohexylurea]. This compound was

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Table I. Recovery of Acetohexamide added to Plasma

Added (mg/dl)	Found (mg/dl)	Recovery (%)
5.0	4.8	96.0
5.0	4.7	94.0
5.0	4.7	94.0
5.0	4.8	96.0
5.0	4.8	96.0
10.0	9.6	96.0
10.0	9.3	93.0
10.0	9.4	94.0
10.0	9.6	96.0
10.0	9.7	97.0

Mean ± SD: 95.2 ± 1.2
CV: 1.3%

also known to be an effective hypoglycemic agent and was determined as acetohexamide by the present method. The amount of acetohexamide plus hydroxyhexamide is expressed in terms of total acetohexamide concentration in the following. A change in the total plasma concentration of acetohexamide obtained from 5 diabetic subjects is shown in Fig. 3. As Fig. 3 depicts, the total acetohexamide concentration reaches the maximum in about 3 hours.

The level of plasma creatinine after a dose (500 mg) of the drug is illustrated in Fig. 4. The maximum concentration was observed in the period 2—3 hours and the change in the level correlated with the plasma concentration of total acetohexamide. It is evident from Fig. 4 that the false increase caused by the drug ranges from about 40 to 100 percent in given patients. Attention should be paid to these results in order that there may be no erroneous interpretation. The reason that acetohexamide value increased falsely the creatinine values was probably ascribable to the chemical reaction of its methylene group with picric acid. Within 5 hours, acetohexamide seemed to be metabolized largely to hydroxyhexamide which

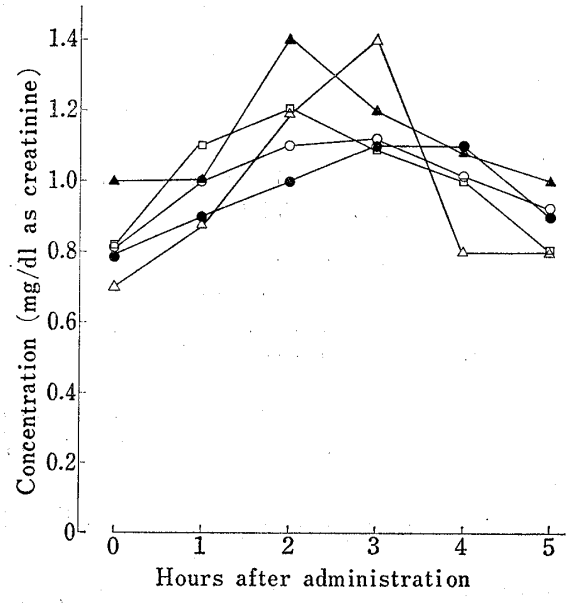

Fig. 4. Changes with Time in Concentration of Plasma Creatinine

The precision of the assay procedure was measured from 10 replicates of a normal pooled plasma. The average creatinine was 1.15 ± 0.02 (SD) mg/dl, with a CV of 1.8%. The symbols are the same as in Fig. 3.

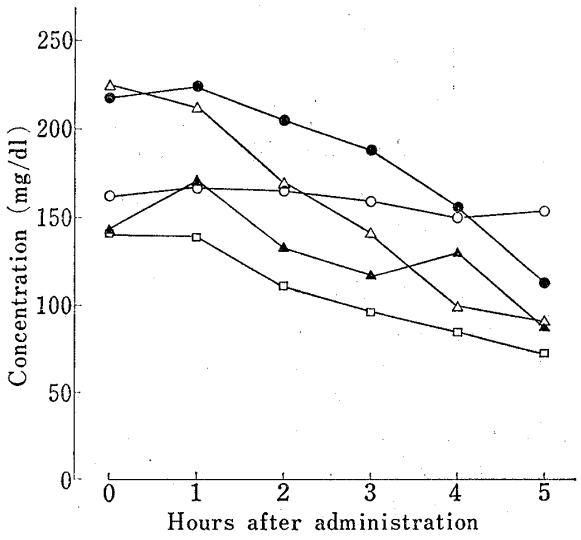

Fig. 5. Changes with Time in Concentration of Plasma Glucose

The symbols are the same as in Fig. 3.

was not responsible for these interferences. The plasma creatinine level returned approximately to its original value as shown in Fig. 4.

The time course observation in plasma glucose was also carried out at an interval of one hour. The result is illustrated in Fig. 5.

The proposed method is superior to the spectrophotometry with DNFB, since the former is not affected by interfering chromogens present in normal drug-free plasma or hemolyzed plasma.