On the other hand, a rabbit which was considered to be under an advanced diabetes showed different results (Fig. 4). After withdrawal of insulin treatment, glucose level markedly exceeded the level before the insulin treatment began. Thus, in rabbits which showed hypoalbuminemia and loss of body weight with abnormally high levels of blood glucose after withdrawal of insulin treatment, a possible existence of complications with diabetes might be considered.

If there are complications with diabetes, analysis of the experimental results in the pharmacokinetic study of drugs in the diseased state will be more complicated. A constant picture of the disease without complications is desired.

Therefore, the above three rabbits which showed hyperalbuminemia and constant body weight with hyperglycemia even after withdrawal of insulin treatment were considered to be at a constant diseased state suitable for the pharmacokinetic study of drugs in alloxan diabetic rabbits.

Thus, it was concluded that alloxan diabetic rabbits suitable for the study for pharmacokinetic behavior of drugs can be selected by confirmation of hyperglycemia, hyperalbuminemia and constant body weight.

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UDC 615.225.2.011.4.074 : 543.426.061

Studies on Ecarazine Hydrochloride (Apiracohl®). II.[1] Spectrofluorometric Determination of Ecarazine Hydrochloride and Its Metabolite in Plasma

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(Received December 12, 1977)

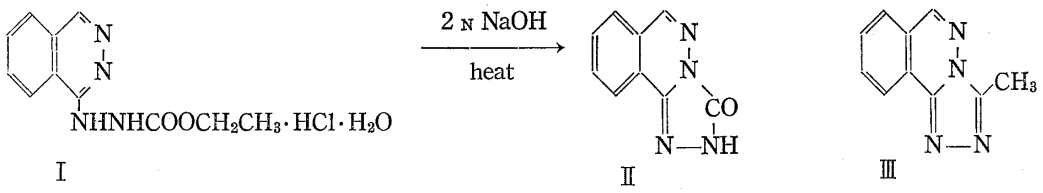
Spectrofluorometric assay for the quantitative determination of ecarazine hydrochloride (I) and its metabolite, 3-methyl-*s*-triazolo(3,4-*a*)phthalazine (III) were described. I and III were extracted from plasma at weakly alkaline pH with chloroform and fluorescence of III was directly measured (Ex 250, Em 410 nm). The chloroform layer containing I was treated with alkali at elevated temperature to give *s*-triazolo(3,4-*a*)phthalazine-2H-3-one (II). The fluorescence of II was measured at an emission wavelength 470 and excitation of 266 nm. There was a linear relationship between concentration of I and fluorometric response up to 10 μg/ml in plasma. This assay was sensitive enough to be useful for the determination of I and III and had a sensitivity limit of 0.2 and 0.07 μg/ml, respectively. Other antihypertensive agents and some metabolites had no detectable effect on the present assay. The method was applied to the determination of plasma levels in rats and dogs after oral or intravenous administration (3 or 10 mg/kg) of I.

Keywords——ecarazine hydrochloride; spectrofluorometry; rat plasma; dog plasma; antihypertensive drugs

Ecarazine hydrochloride (N_1-Carbethoxy-N_2-hydrazinophthalazine hydrochloride, I), a derivative of hydralazine, is well established as a therapeutic agent for the treatment of essential hypertention. In the previous paper,[1] I was found to be metabolized to some extent to hydralazine. In hydralazine therapy, systemic lupus erythematosus (S. L. E.) has been observed as a serious side effect, and a high plasma drug concentration was attributed

1) Part I: A. Ishii, T. Deguchi and H. Takahira, *Yakugaku Zasshi*, 93, 1383 (1973).
2) Location: *1188 Shimotogari, Nagaizumi machi, Sunto gun, Shizuoka, Japan.*

to crucial factor both in the appearance of the syndrome[3] and in the magnitude of its hypotensive effect.[4] From these aspects and because of individual variation in metabolism, the plasma concentrations of I and its metabolite must be determined. Several colorimetric methods have been developed for the estimation of I in a pure form and in tablets.[5–8] These methods, however, were neither sufficiently sensitive nor specific to monitor the drug concentration in biological fluids. In the present paper, a sensitive spectrofluorometric method is developed for the determination of *s*-triazolo(3,4-*a*)phthalazine-2H-3-one (II) derived from I and 3-methyl-*s*-triazolo(3,4-*a*)phthalazine (III), a major metabolite[1] (see Chart 1). The present method is applied to estimate the plasma levels in rat and dog after administration of I.



I II III

Chart 1

Experimental

Materials——Ecarazine hydrochloride used was a product of Kyowa Hakko Kogyo. *s*-Triazolo(3,4-*a*)-phthalazine-2H-3-one, 3-Methyl-*s*-triazolo(3,4-*a*)phthalazine, *s*-Triazolo(3,4-*a*)phthalazine, and 1-(2H)phthalazinone were prepared by the methods of Ishii,[1] Druey,[9] Potts,[10] and Gabriel,[11] respectively. All reagents were of analytical grade and used without further purification. All aqueous solutions were prepared using distilled deionized water. Hydralazine, guanethidine sulfate, furosemide, L-α-methyldopa, propranolol hydrochloride, reserpine, and trichlormethiazide were obtained from various commercial sources. These chemicals were of the highest purity available, and used without further purification.

Animals——Male Wistar rats weighing 200±10 g were orally administered with 2 ml of ecarazine hydrochloride solution (3 or 10 mg/kg). Four dogs (male 10 kg) were anesthetized with sodium pentobarbital (30 mg/kg *i.v.*), then I was administered intravenously (3 or 10 mg/kg). At 5, 10, 15, 30, 60, 90, 120 min after injection, groups of 5 rats were decapitated and exsanguinated. The blood samples (heparinized) were immediately chilled in ice and centrifuged at 1000 *g* for 10 min. Analysis was carried out within 24 hr.

Assay Procedure——I was determined by the following method. A 1.0 ml portion of plasma was taken in a glass-stoppered 10 ml centrifuge tube, adjusted to pH 9 with 0.1 N NaOH, shaken with 5 ml of chloroform for 30 min and centrifuged at 3000 rpm for 10 min. The chloroform phase was transferred to another tube and evaporated to dryness. The dry residue was dissolved in 1 ml of 2 N NaOH and heated in a boiling water for 90 min. The solution was cooled to room temperature and adjusted to pH 6.5 with 2 N HCl. The acidified solution was shaken with 5 ml of chloroform for 30 min and centrifuged at 3000 rpm for 10 min. After the aqueous phase was removed by aspiration, the fluorescence of remaining chloroform phase was measured in a 1-cm quartz cell at 470 nm, excitation at 266 nm by a Hitachi spectrofluorometer model MPF-2A equipped with a Xenon lamp. The entire procedure was carried out for plasma samples containing known amounts of I along with the test samples.

The concentration of III in plasma was determined by the modified method of Reidenberg.[12] The extraction of III was carried out with the same method as the first step (extraction at pH 9) for I. The chloroform layer was measured at 410 nm with excitation at 250 nm. The concentrations of III in the samples were estimated by reference to the standard solutions of III in plasma prepared simultaneously.

3) H.K. Perry, Jr., E.M. Tan, S. Carmody and A. Sakamoto, *J. Lab. Clin. Med.*, **76**, 114 (1970).
4) R. Zacest and J. Koch-Weser, *Clin. Pharmacol. Ther.*, **13**, 420 (1972).
5) T. Cieszynski, *Acta Polon. Pharm.*, **25**, 551 (1968).
6) T. Cieszynski, *Acta Polon. Pharm.*, **25**, 613 (1968).
7) Z. Modras. *Farm. Pol.*, **5**, 347 (1969).
8) H. Ellert and Z. Modras, *Farm. Pol.*, **7**, 511 (1971).
9) J. Druey and B.H. Ringer, *Helv. Chim. Acta*, **34**, 195 (1951).
10) K.T. Potts and C. Lovelette, *J. Org. Chem.*, **34**, 3221 (1969).
11) S. Gabriel, *Ber.*, **26**, 521 (1893).
12) M. Reidenberg, D. Drayer, J. Martin, A.L. De Marco and C.T. Bellow, *Clin. Pharm. Ther.*, **14**, 971 (1973).

Test for Drug Interferences——Some commercially available antihypertensive drugs were examined for possible interference at the therapeutic concentrations. The drugs were individually mixed with aliquots of normal plasma and assayed for I following the procedure described above.

Results and Discussion

Properties of the Fluorescent Product

A fluorometric method was already reported for the quantitative determination of III in urine (Ex 320, Em 420 nm).[12] However, high sensitivity has not been obtained in plasma because of high blank fluorescence (Ex 355, Em 470 nm). In our experiment III showed native fluorescence with 3 distinct excitation maxima at wavelengths 250, 280 and 320 nm and emission maximum at 410 nm. Excitation at 250 nm gave more specific and sensitive spectrum for III than at 280 and 320 nm as shown in Fig. 1. The detection limit of the present method was 0.07 μg/ml in plasma and at this concentration the fluorescence was twice that of plasma blank.

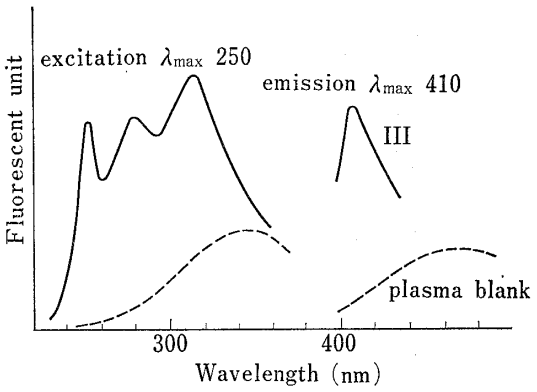


Fig. 1. Excitation and Emission Spectra of 3-Methyl-*s*-triazolo(3,4-*a*)phthalazine (III)

Emission spectra of III and plasma blank were obtained at the excitation wavelengths of 250 and 355 nm, respectively.

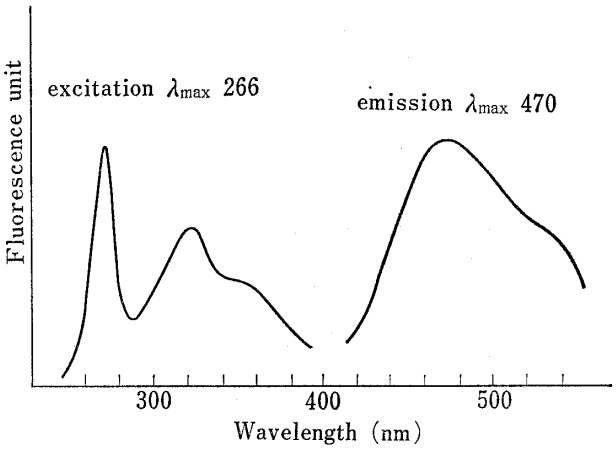


Fig. 2. Excitation and Emission Spectra of the Fluorescent Product (II) from Ecarazine Hydrochloride (I)

An intensively fluorescent product was obtained from I in 2 N NaOH solution by heating in a boiling water bath. In order to separate the fluorescent product from interfering materials presented in plasma, the extractability of fluorescent product in pH range of 3 to 8 was studied. I was quantitatively extracted with chloroform from aqueous layer and background fluorescence was minimum at pH 6.5. II exhibited strong fluorescence in halogenated solvents *e.g.*, chloroform, ethylene dichloride and methylene dichloride. The excitation and emission spectra of this compound are shown in Fig. 2. II exhibited excitation at 266 and 320 nm and a broad emission maximum at 470 nm. All measurements were carried out at Ex 266 nm because of low plasma blank. The reaction product thus obtained was stable in an alkaline solution for 7 hr (90% sustained). However at the first chloroform extraction step (weakly alkaline pH), II was not produced. Therefore the fluorescence of II does not interfere with the determination of III. On the contrary, III was very unstable in a strongly alkaline solution and was derived to 2-methyl-5-(2-cyanophenyl)-*s*-triazole (Ex 265, Em 330 nm) by rearrangement,[13] and its relative intensity was 0.094 to the fluorescence product from I (see Table I). Therefore III interfered with the determination of I at the same

13) A.W. Pruitt and P.G. Dayton, *Eur. J. Clin. Pharm.*, 4, 59 (1971).

concentration levels and the net concentration of I in biological samples was calculated by subtracting the contribution (9.4%) of III.

The fluorescence of II was found to be linearly correlated with concentration of II over the range of 0.04—0.67 μg/ml in chloroform (see Fig. 3). The relative standard deviations were 0.7, 1.7, 5.9 and 5.5% for the standard solution of 0.625, 1.25, 2.5 and 5 μg/ml in plasma, respectively. A linear relationship was observed between concentration of I and fluorometric response up to 10 μg/ml in plasma samples (see Fig. 4). The sensitivity limits for I was 0.20 μg/ml using 1 ml plasma per assay and a sample-blank fluorescence ratio was 2: 1. This value provided 50 fold increase in sensitivity over the colorimetric methods.[5–8]

Table I. Fluorescence Properties of Alkali Hydrolysis Products of Antihypertensive Drugs and Some Metabolites of Ecarazine Hydrochloride

Compound[a]	Fluorescence[b] maxima in $CHCl_3$ (Ex/Em, nm)	Fluorescence[c] (arbitrary units)
Ecarazine hydrochloride (I)	266, 320/470	154.5
3-Methyl-*s*-triazolo(3,4-*a*)phthalazine (III)	265/330	14.5
s-Triazolo(3,4-*a*)phthalazine (IV)	265/320	14.0
1-(2H)Phthalazinone	290/350	3.0
Reserpine	280/340	5.0
Furosemide	350/395	0.5
Hydralazine	—[d]	0
Guanethidine sulfate	—	0
Propranolol hydrochloride	—	0
L-α-Methyldopa	—	0
Trichlormethiazide	—	0

a) Compound submitted to alkali hydrolysis at the plasma level of 10 μg/ml.
b) By the assay procedure of I.
c) At Ex 266, Em 470 nm.
d) No fluorescence.

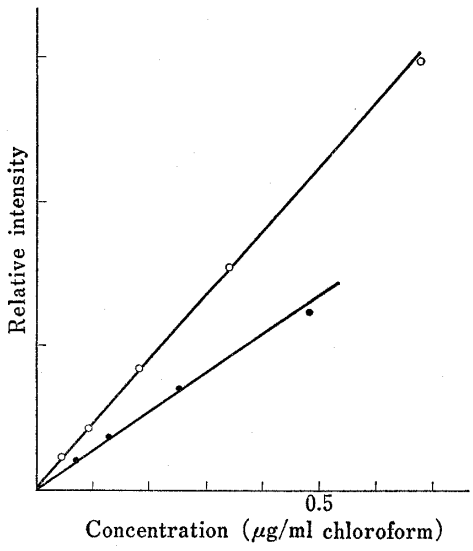


Fig. 3. Fluorometric Standard Curves of II and III in Chloroform Solution

—○—; II, —●—: III.

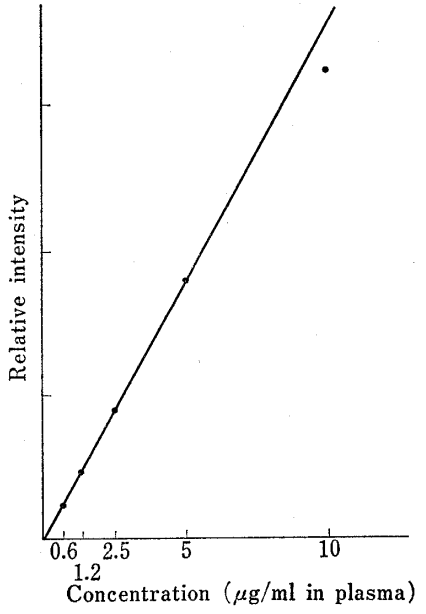


Fig. 4. Fluorometric Standard Curve Obtained for I (determined as II) in Plasma Samples

Interference by other metabolites of I was studied on the fluorometric assay of III. Although *s*-triazolo(3,4-*a*)phthalazine (IV, Ex 250, 275 and 320, Em 390 nm) was observed to interfere at the same concentration level as III, the fluorescence spectrum of plasma administered with I following the assay procedure of III showed very similar pattern to III. This indicated the concentration of IV was negligible in plasma. Interference of another metabolite, 1-(2H)phthalazinone(Ex 290, Em 350 nm), with the assay of I was examined. The intensity of this metabolite relative to I was 0.019 (see Table I). Several drugs that might be used in combination with I were tested to see if they disturbed the assay and the results are shown in Table I. Reserpine and furosemide showed a little fluorescence (the relative intensities were 0.032 and 0.003 to I, respectively). The other drugs did not form any detectable amount of fluorescence.

Plasma Concentration

The plasma levels of I and III were determined in dogs and rats following administration of a single dose of I. III is formed by acetylation and subsequent cyclization of hydralazine and excreted mainly in urine.[14] The plasma concentrations of I and III were shown in Figs. 5 and 6. In rats, absorption of I was rapid and the maximal concentration was attained at 5—15 min. The peak levels were 0.3 and 1.8 μg/ml at the dose of 3 and 10 mg/kg (*p.o.*), respectively. The half life was 35 min from curve A (see Fig. 5). The concentration of metabolite III was gradually increased and reached 0.46 μg/ml at 60 min after the administration of 10 mg/kg. In dogs, I also rapidly disappeared from plasma with the half life of 30—40 min as in rats (see Fig. 6).

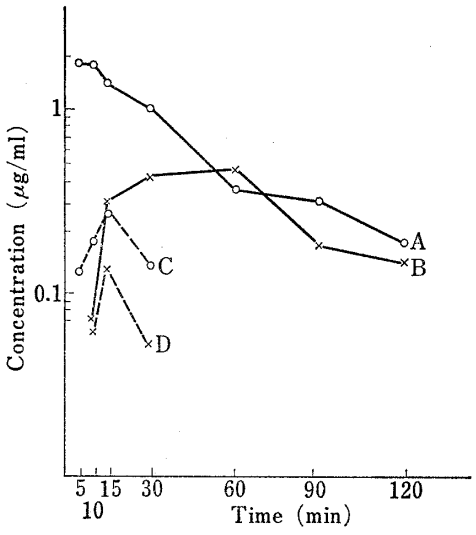


Fig. 5. Plasma Levels of I and the Metabolite III in Rats after Oral Administration of I

Curve A; I, B; III) 10 mg/kg.
C: I, D: III) 3 mg/kg.

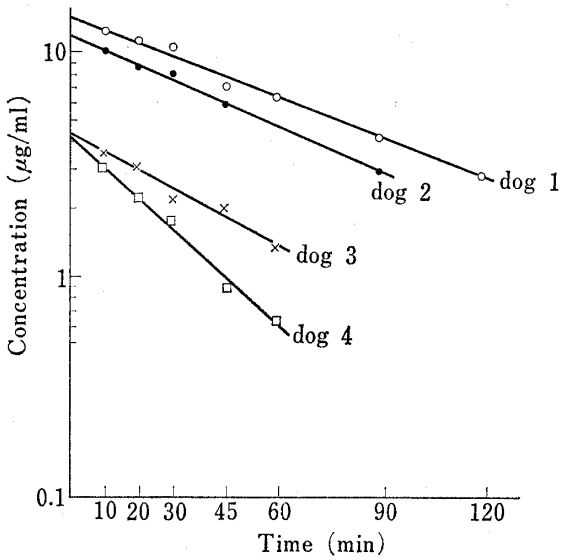


Fig. 6. Plasma Levels of I in Dogs after Intravenous Administration of I

—○—, —●—: 10 mg/kg.
—×—, —□—; 3 mg/kg.

The fate of I was investigated in rats with an aid of 1-^{14}C-ecarazine hydrochloride as described in the previous paper.[1] The plasma levels in radioactivity were 1.01 (3 mg/kg) and 8.61 μg/ml (10 mg/kg) at 30 min after oral administration of I in SHR (male Wistar, 320—350 g, n=3). Until 30 min after the administration, I already accounted for a small

14) H. Zimmer, J. McManus, T. Novinson, E.V. Hess and A.H. Litwin, *Arzneim. Forch.*, **20**, 1586 (1970).

fraction of circulating radioactivity according to the result of thin-layer chromatography (Wakogel B5FM cyclohexane: acetone 1:1). Content of unchanged drug I (1.02 μg/ml at 30 min) accounted for about 12% of circulating radioactivity (8.61 μg/ml), indicating that I underwent extensive metabolism. We found 1-^{14}C-ecarazine hydrochloride given orally was well absorbed by rats, and 85.5% of the administered radioactivity was recovered in urine within 48 hr.[1] Only a small amount of unchanged drug was recovered in urine, indicating I was further transformed in rat. The applicability of the present method to the determination of urine and tissue levels of I is currently investigated.

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Synthesis of Formamidines from Carbodiimides with Sodium Borohydride in Isopropanol

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Reduction of the N,N′-disubstituted carbodiimides with sodium borohydride in isopropanol performs a convenient synthetic method of the corresponding formamidines.

tert-Butanol, pyridine, and tetrahydrofuran proved also available as solvents for the procedure, while methanol or ethanol was unsuitable for the lower yield of the formamidine, probably due to the further reduction to proceed.

Keywords——sodium borohydride; formamidines; carbodiimides; reduction; isopropanol

In connection with the new synthesis of formamidines from the 1,3-disubstituted ureas with sodium borohydride in pyridine,[2] we have investigated the reduction of carbodiimides to give the corresponding formamidines. Readily available sodium borohydride was employed for the reduction of carbodiimides to obtain formamidines under simple operating conditions, although the catalytic hydrogenation of carbodiimides had already been reported as a useful synthetic method.[3]

The reduction of N,N′-dicyclohexylcarbodiimides (**1a**) with sodium borohydride carried out at 50° for 4 hr in methanol and ethanol which are quite commonly available solvents in borohydride reduction, and N,N′-dicyclohexylformamidine (**2a**) was obtained in low yields (19% and 50% respectively). Unexpectedly shortening the reaction time (30 min, 30°, EtOH) raised the yield of **2a** to 88%. The explanation of the results is that the formamidine formed in the reaction mixture is unstable under this condition and decompose gradually during the reduction,[4] however the exact reason explaining the results is still obscure.

On the other hand, in isopropanol N,N′-dicyclohexylformamidine (**2a**) was obtained in a good yield as shown in Table I and no extensive decomposition of **2a** or the formation of significant side products was not observed even with 8 hr stirring.

1) Location: *Mitahora, East 5-6-1, Gifu 502, Japan*; *a*) To whom inquiries should be adressed; *b*) *Keyakidai, 1-1, Sakado, Saitama, 350-02, Japan.*
2) Y. Kikugawa, S. Yamada, H. Nagashima, and K. Kaji, *Tetrahedron Lett.*, **1969**, 699.
3) J.C. Jochims, *Chem. Ber.*, **98**, 2128 (1965).
4) Several spots other than that of **2a** were detected by the thin-layer chromatography of the reaction mixture.