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Studies on Heart. XXIII.[1] Distribution of [1-^{14}C] Acetamidino-antiarrhythmic Peptide (^{14}C-AAP) in Mice[2]

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The distribution of [1-^{14}C] acetamidino-antiarrhythmic peptide (^{14}C-AAP) in mice was studied by direct counting and whole-body autoradiography. Intravenously injected ^{14}C decreased rapidly in blood during 1 to 20 min and gradually during 20 to 180 min. At 180 min 68% of the radioactivity had appeared in the urine. At 60 min unmetabolized ^{14}C-AAP was present in the urine. At 1 min the radioactivity was highest in the kidney, followed by the lung, liver, and myocardium in that order. Radioactivity increased in the kidney and myocardium during 1 to 6 min, while the radioactivity in the lung and liver decreased rapidly from 1 to 60 min. Autoradiograms revealed that at 6 min after injection (*i.v.*) the highest radioactivities were seen in the kidney, urine, heart, lung, submaxillary gland, bone and gut wall. No radioactivity was present in the brain, spinal cord, gut contents or fetus. At 20 to 60 min the radioactivity levels in the organs were much less than those seen at 6 min, except for the kidney and urine. In mice orally given ^{14}C-AAP, the maximum plateau levels of radioactivity were found in the myocardium (0.04% of dose) and blood (2.5%) at 40 to 120 min. In serum at 60 min, ^{14}C-AAP was still present in the original molecular form, unbound to macromolecules.

Thus, ^{14}C-AAP accumulates in the myocardium of the target organ for a short time immediately after injection (*i.v.*), corresponding to the period of the QTc interval prolongation by AAP. ^{14}C-AAP does not accumulate for long in any organ except for the kidney, and is largely excreted in urine within 60 min.

Keywords——peptide; antiarrhythmic peptide; ^{14}C-labeled peptide; arrhythmia; distribution; whole-body autoradiography

In connection with our studies on the humoral factors affecting heart functions, we have reported in our previous paper[3] the isolation of an atrial peptide (AAP) which improves the rhythmicity of cultured myocardial cells. AAP was found to be a new hexapeptide, Gly-Pro-Hyp-Gly-Ala-Gly,[4] and to have antiarrhythmic effects almost equal in intensity to those of quinidine in experimental aconitine-, $CaCl_2$- and ouabain-induced arrhythmias in dogs, rats and mice.[1] In this paper, as a first approach to achieve an understanding of the action mechanism, the toxicity and the behavior of AAP *in vivo,* this peptide was synthesized by the solid phase method, and the distribution of the ^{14}C-labeled compound was determined in mice.

Experimental

Materials——Aconitine was product of Sigma Chemical Company. Ethyl [1-^{14}C] acetimidate hydrochloride (specific activity : 57.5 mCi/mmol) and liquid scintillation cocktail, ACS II, were products of Amersham. Boc-amino acids, their derivatives, dicyclohexylcarbodiimide (DCCD), anhydrous HF, chloromethylated polystyrene (divinylbenzene 2 %, 200—400 mesh, Cl : 1.3 meq/g), trifluoroacetic acid (TFA) and aminopeptidase M (APM) were products of the Protein Research Foundation. All other reagents and solvents used for peptide synthesis were special grade products of Nakarai Chemical Ltd. CH_2Cl_2 was distilled after being dried with anhydrous $CaCl_2$. Triethylamine (TEA) was dried with NaOH granules, refluxed with 2% ninhydrin and distilled.

Animals——Animals were male ddy mice weighing 20—25 g and female ddy mice at the 18th day of pregnancy.

Analytical Procedures——The samples for radioactivity measurement were placed in counting vials and 10 ml of ACS II was added. The radioactivity was counted in a liquid scintillation spectrometer (Beckman, LS-150). In the ^{14}C analyses of biological samples, the apparent counts were corrected by an internal standard method. Paper chromatography was carried out on Whatman 3MM paper strips using solvent I, ethanol/water (63 : 37), and solvent II, *n*-butanol/pyridine/acetic acid/water (15 : 10 : 3 : 12). Staining was carried out with KI-starch or ninhydrin reagent. In the case of radioactive samples, the cut paper strips were counted. APM digestion was carried out by incubating 0.2 ml of sample (0.4 μmol) with 0.2 ml of 0.1% enzyme solution at 37°C for 72 h. Amino acid analysis, dansyl-Edman degradation and measurement of rotation were carried out as described previously.[4]

Solid Phase Synthesis of AAP——A solution of Boc-Gly (22 g, 126 mmol) and TEA (17.5 ml, 126 mmol) in ethanol (133 ml) and $CHCl_3$ (67 ml) was stirred with the resin (50 g) at room temperature for 1 h and then at 90°C under reflux for 48 h. The resin was filtered off, washed with ethanol, acetic acid, water and methanol successively, and dried *in vacuo* under 30°C. Amino acid analysis of an acid hydrolysate[5] of Boc-Gly-resin gave Gly corresponding to 0.364 mmol/g resin. Boc-Gly-resin (55.7 g, 20.27 mmol Gly) was placed in a reaction vessel (700 ml, Kato Shoten), and the following cycle of deprotection, neutralization and coupling was carried out by shaking at 10 cycles/min in 350 ml of solution for the introduction of each new residue[6]: 1) 3 washings with CH_2Cl_2 for 3 min, 2) cleavage of the Boc group by treatment with 50% TFA/CH_2Cl_2 for 3 and 20 min, twice, 3) 8 washings with CH_2Cl_2 for 3 min, 4) neutralization of TFA with 10% TEA in CH_2Cl_2 for 3 and 10 min, twice, 5) 5 washings with CH_2Cl_2 for 3 min, 6) addition of 60.81 mmol of the Boc-amino acid in CH_2Cl_2 (250 ml) and mixing for 10 min, 7) addition of 60.81 mmol of DCCD in CH_2Cl_2 (100 ml) followed by a reaction period of 2 h and 8) 5 washings with CH_2Cl_2 for 3 min. Thus, Boc-Ala, Boc-Gly, Boc-Hyp(OBzl), Boc-Pro and Boc-Gly were introduced successively onto the Boc-Gly-resin. After the introduction of the final residue, the peptide-resin was washed with CH_2Cl_2, ethanol, acetic acid and CH_2Cl_2 successively, and dried *in vacuo* at below 30°C (yield : 68 g). According to the method of Sakakibara et al.,[7] the peptide-resin (about 10 g) was placed in an Hydrogen feuoride (HF) reaction vessel (HF-reaction apparatus, Type II, Protein Research Foundation) and mixed with anisole (10 ml). Anhydrous HF (100 ml) was then introduced into the vessel, and the mixture was allowed to react at 0°C for 1 h. After the excess HF had been evaporated off *in vacuo*, the generated free peptide was extracted with water. The extract was washed with ether and lyophilized, (yield : 10 g). A 1—2 g portion of the crude product was applied to a Sephadex G-15 column (3.5×85 cm) and eluted with 0.1M acetic acid. The eluates at 360—444 ml elution volume, which gave a UV absorption peak at 235 nm, were combined and lyophilized. The dried material was dissolved in water, adjusted to pH 2 and applied to an Amberlite CG-120 column (Na form, 1.8×150 cm), which was eluted with 0.20M sodium citrate buffer (pH 3.25) at 55°C at 120 ml/h. A ninhydrin-positive peak which was eluted between 820 and 900 ml, was collected and applied to a Dowex 50W column (Na form, 4.6×25 cm) equilibrated with 0.01N HCl. The column was developed with 1000 ml each of 0.01N HCl and 2N NH_3. The eluate with 2N NH_3, which showed a ultraviolet (UV) absorption peak at 235 nm, was evaporated and lyophilized repeatedly to give AAP, (yield: 5 g, $[\alpha]_D^{20}$: −133° (c=1, 0.5N HCl), amino acid ratio: Gly, 3.00; Ala, 0.99; Hyp, 0.93; Pro, 0.99 by 6N HCl, 110°C, 24 h, Gly, 3.00; Ala, 1.01; Hyp,0.46; Pro, 0.29 by APM, 37°C, 72 h, amino acid sequence: Gly-Pro-Hyp-Gly-Ala-Gly by dansyl-Edman degradation, *Anal.* Calcd for $C_{19}H_{30}N_6O_8$ (470): C, 48.51; H, 6.38; N, 17.78. Found: C, 48.14; H, 6.42; N, 17.73.

Preparation of ^{14}C-AAP——AAP was labeled with ethyl [1-^{14}C] acetimidate according to the method of Hunter *et al.*[8] AAP (4.1 mg, 8.7 μmol) was incubated with ethyl [1-^{14}C] acetimidate hydrochloride (0.5 mCi, 8.7 μmol) in 190 μl of 0.2M sodium borate buffer (pH 9.0) at 25°C for 1 h. The reaction mixture was adjusted to pH 3—4, applied to a Sephadex G-10 column (1.2×145 cm) and developed with 0.02N acetic acid. The fractions at 50—65 ml elution volume, which showed radioactivity and UV absorption at 235 nm, were collected, adjusted to pH 2 and applied to an Amberlite CG-120 column. The column was run under the same conditions as described for the purification of AAP. The fractions at 576—600 ml elution volume, which showed a peak with radioactivity and ninhydrin color after alkaline hydrolysis,[9] were combined, concentrated by lyophilization, desalted with a Sephadex G-10 column (3×86 cm) and lyophilized to give ^{14}C-AAP, (yield: 4.1 mg, specific activity: 104 μCi/mg). In a separate experiment, 51 mg of AAP was labeled with ethyl acetimidate and purified in the same way, (yield: 50 mg, amino acid ratio: Gly,3.00; Ala, 0.96; Hyp, 1.08; Pro, 0.93 by 6N HCl, 110°C, 24 h, $[\alpha]_D^{20}$: −122° (c=1, 0.05N HCl), *Anal.* Calcd for $C_{21}H_{33}N_7O_8$ (511): C, 49.30; H, 6.46; N, 19.20. Found: C, 49.57; H, 6.28; N, 19.28). Acetamidino-Gly was also prepared in the same way.

Antiarrhythmic Activity——Mice were anesthetized with sodium pentobarbital (45 mg/kg, *i.p.*). Aconitine (7.5 μg/ml) was infused according to the method of Nwangwu *et al.*,[10] at a constant rate of 0.25 ml/min using an infusion pump (Nihon Koden, TFV-1100) from 3 min after sample injection. The sample or aconitine was administered *via* a cannula into the femoral vein. The onset time of atrio-ventricular (A-V) block or ectopic beat was measured as an end point by electrocardiography (ECG, lead II) using a Polygraph system (Nihon Koden, RM-6000).

^{14}C Analyses in Mice Given ^{14}C-AAP——After ligation of the penis, each mouse received a single injection of 1 μCi of ^{14}C-AAP in 0.1 ml of solution *via* a femoral vein. The mice were anesthetized lightly with ether after the prescribed time intervals. Blood, myocardium, lung, kidney, a lobule of liver and urine were

removed. In addition, blood and myocardium of mice orally given 1 μCi of ^{14}C-AAP were removed. Organs were freed from fat and other tissues and rinsed with saline to exclude blood. The samples for radioactivity measurement were placed in counting vials, then 0.4 ml of 60% perchloric acid-30% H_2O_2 (1:1) was added and the sealed vials were heated at 80°C for 2 h.[11] The 60 min urine from mice intravenously injected with ^{14}C-AAP was analyzed for radioactivity by paper chromatography and ion exchange chromatography. The serum separated from blood 60 min after oral administration of ^{14}C-AAP was eluted from a Sephadex G-25 column (2.0×92 cm) with phosphate-buffered saline (pH 7.3). The radioactive peak was analyzed by paper chromatography. A serum sample also was analyzed by ion exchange chromatography. Independently, mice were given a single injection of 8 μCi of ^{14}C-AAP in 0.1 ml of solution *via* a femoral vein. The mice were anesthetized lightly with ether and frozen by immersion in dry ice and acetone at either 6, 20 and 60 min after injection. Frozen whole-body sagital sections, 40 μm thick, were taken on Sellotape RI-50 (Hisamitsu Seiyaku) using a microtome (Leitz 100) and allowed to dry for 10 days in a freezer. Autoradiograms were prepared by placing the frozen sections against Fuji X ray film RX for appropriate periods of time.

Results

The ^{14}C-AAP used was of acceptable purity, as shown in the scintigrams in Fig. 1. The cold acetamidino-AAP retained antiarrhythmic activity in the experimental aconitine-induced arrhythmia of mice, as shown in Table I. The potency of acetamidino-AAP was almost equal to that of AAP.

Direct Counting of Organs, Blood and Urine

Intravenously injected ^{14}C decreased rapidly in the blood during 1 to 20 min after injection, and decreased gradually during 20 to 180 min (Fig. 2). On the assumption that the radioactivity reaches diffusion equilibrium in the body fluid, at 6 min after injection the blood

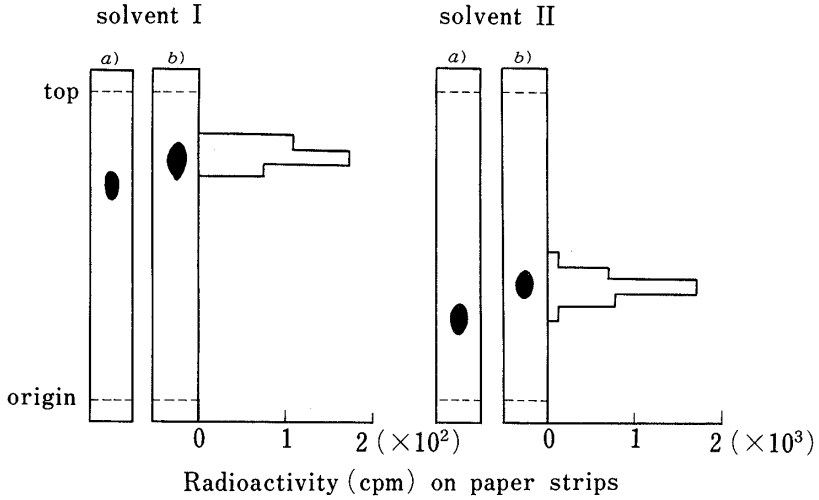

Fig. 1. Scintigrams Obtained by Paper Chromatography of ^{14}C-AAP

a) AAP, *b*) ^{14}C-AAP. Staining, KI-starch (dark area); solvent I, ethanol/water (63:37); solvent II, *n*-butanol/pyridine/acetic acid/water (15:10:3:12).

TABLE I. Effect of Acetamidino-AAP on Aconitine-induced Arrhythmia in Mice

Sample[a)]	Dose (mg/kg, *i. v.*)	No. of mice	Onset of initial arrhythmia[b)] (s)
Saline	—	8	95.9±2.8
Acetamidino-AAP	25	8	121.4±9.5[c)]
AAP	25	8	125.0±3.4[d)]

a) Sample was injected 3 min before aconitine infusion.
b) Onset of atrio-ventricular block or ectopic beat was monitored. Data are means ± S. E.
c) $p<0.05$, *d*) $p<0.001$: *versus* saline.

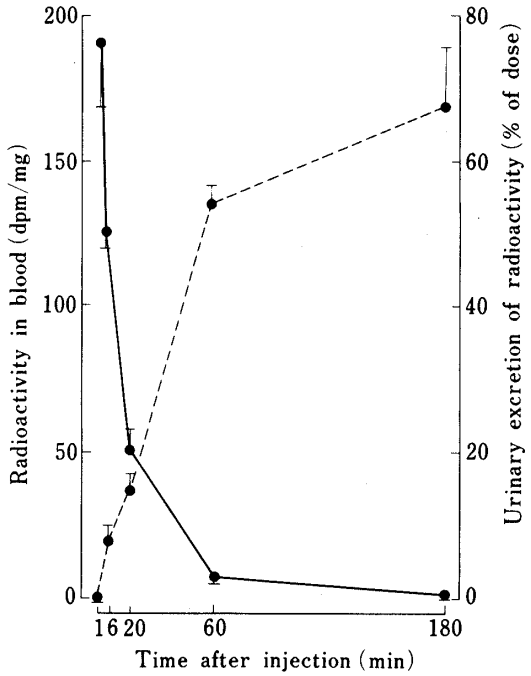

Fig. 2. Blood Level and Urinary Excretion of ^{14}C after Intravenous Injection of ^{14}C-AAP into Mice

Each point shows the mean±S.E. of 4 mice.

level of radioactivity should be about 65% of the expected concentration (200 dpm/mg blood) at 0 time, and its half-life in blood should be about 10 min. At 6 min after injection, 8% of the radioactivity dosed had been excreted in urine (Fig. 2). The excretion of radioactivity in urine increased with decrease of radioactivity in the blood, reaching 68% of the dose at 180 min.

Direct counting of organs after intravenous injection of ^{14}C-AAP revealed subtle but significant changes in the distribution of radioactivity. As shown in Fig. 3, at 1 min after injection the radioactivity was distributed most markedly in the kidney, followed by the lung, liver, and myocardium in that order. The radioactivity in the lung and liver decreased rapidly almost in parallel with the decrease of blood radioactivity. However, the radioactivity in the kidney and myocardium did not dicrease during 1 to 6 min after injection. During 6 to 180 min after injection the radioactivity in myocardium decreased, as did those of the liver and lung, and the radioactivity in the kidney also decreased, but remained much higher than the blood level, reflecting the excretion of radioactivity into the urine.

Blood and myocardium levels of ^{14}C after oral administration of ^{14}C-AAP to mice are shown in Fig. 4. At 20 min after administration the radioactivity was present in blood and myocardium. The maximum plateau of distribution was found in blood (2.5% of dose) and myocardium (0.04% of dose) at 40 to 120 min, and at 180 to 360 min the distributions declined.

Autoradiographic Distribution of ^{14}C-AAP

Autoradiograms (Fig. 5) revealed that at 6 min after injection of ^{14}C-AAP in male mice the highest concentration of radioactivity was seen in the kidney, followed by the urine, heart, lung, submaxillary gland, bone, the wall of the stomach and of the intestine and blood, in that order. Most of the radioactivity in the heart was found to be due to that of blood. No radioactivity was present in the brain, spinal cord and the contents of the stomach and intestine. At 20 min after injection (Fig. 6-A) the amounts of radioactivity in the organs, tissues and blood (except for the kidney and urine) were much less than those at 6 min. At 60 min (Fig. 6-B) no radioactivity remained in the blood, organs, or tissues, except for the kidney and urine. At 20 min after injection into a pregnant mouse the distribution of radioactivity was similar to that in male mouse, and no radioactivity was detected in the fetus.

Analysis of Radioactivity in Serum after Oral Administration

The serum sample separated from blood at 60 min, when the radioactivity in the blood was maximum after oral administration of ^{14}C-AAP (Fig. 4), was fractionated on a Sephadex G-25 column, as shown in Fig. 7. Only one radioactive peak, eluate S, which appeared at K_{av} 0.32, was found, but no radioactivity was detected in the protein peak which showed absorption at UV 280 nm. Eluate S was analyzed for radioactivity by paper chromatography, as shown in Fig. 8. In the scintigrams using two solvent systems, only one radioactive peak, which coincided with ^{14}C-AAP, was detected. Furthermore, as shown in

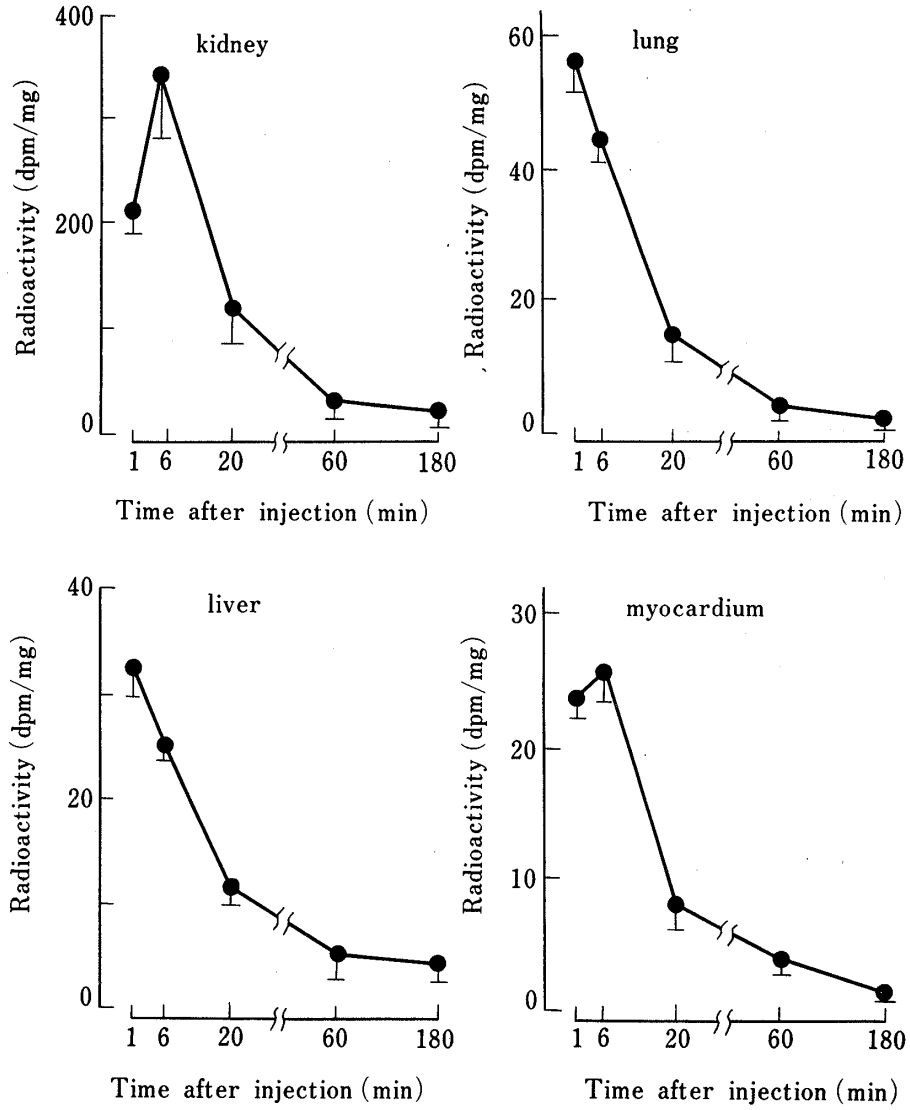

Fig. 3. Organ Distribution of ^{14}C after Intravenous Injection of ^{14}C-AAP into Mice

Each point shows the mean±S.E. of 4 mice.

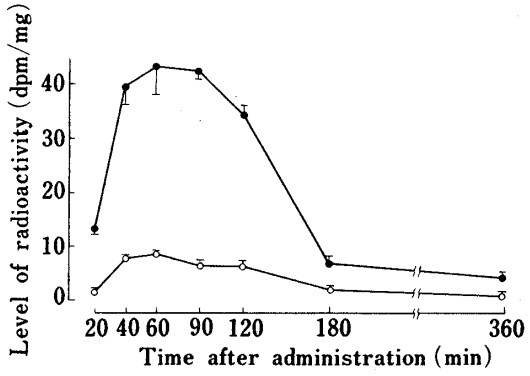

Fig. 4. Blood and Myocardium Levels of ^{14}C after Oral Administration of ^{14}C-AAP to Mice

Each point shows the mean±S.E. of 4 mice.
●, blood; ○, myocardium.

Fig. 9, when the radioactive serum was run on an ion exchange resin, only one radioactive peak which appeared at the same retention time (2 h 6 min) as pure ^{14}C-AAP was developed. That is to say, in the serum of mice 60 min after oral administration of ^{14}C-AAP, ^{14}C-AAP must be present in the original molecular form, unbound to macromolecules.

Analysis of Radioactivity in Urine after Intravenous Injection

Urine samples of mice within 60 min after intravenous injection of ^{14}C-AAP were also analyzed for radioactivity. The radioactivity in the urine gave a single peak,

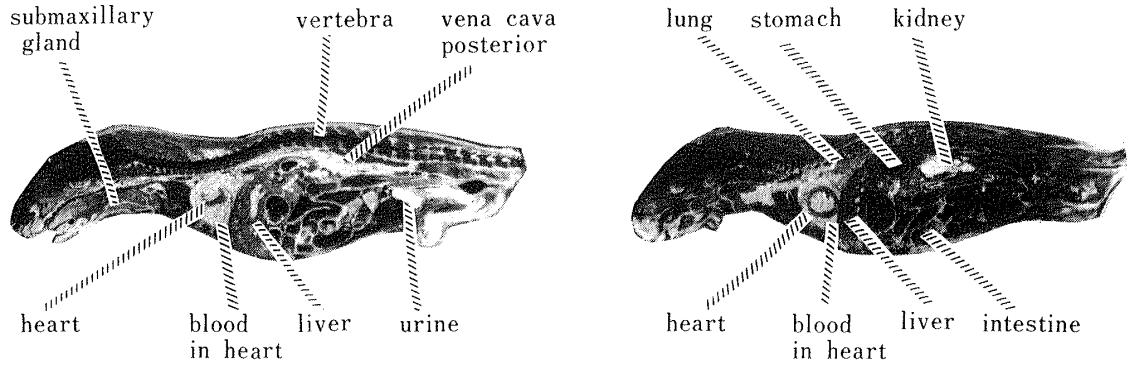

Fig. 5. Whole-body Autoradiograms of Mouse at 6 min after Intravenous Injection of ^{14}C-AAP

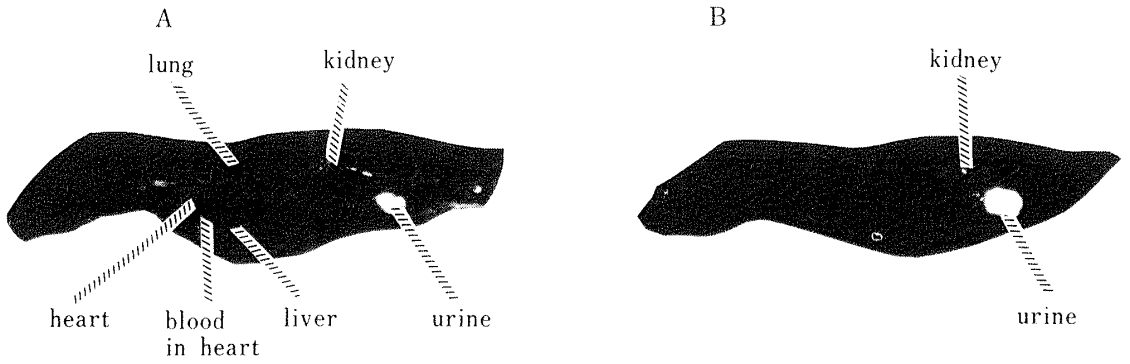

Fig. 6. Whole-body Autoradiograms of Mice at 20 and 60 min after Intravenous Injection of ^{14}C-AAP

A, 20 min; B, 60 min.

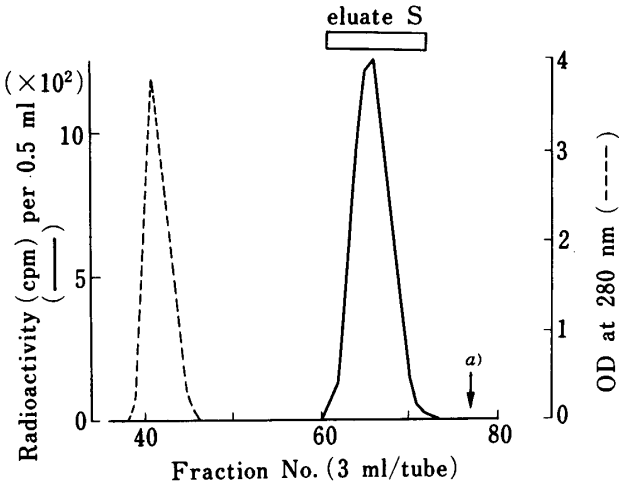

Fig. 7. Gel Filtration of Serum at 60 min after Oral Administration of ^{14}C-AAP to Mice

Column, 1.8×110 cm; solvent, phosphate-buffered saline (pH 7.3).
a) An arrow shows the bed volume measured by applying NaCl solution.

which coincided with that of ^{14}C-AAP, in paper chromatography (Fig. 8) and ion-exchange chromatography (Fig. 9). About 55% of intravenously injected ^{14}C-AAP must be excreted into the urine without being metabolized within 60 min after injection.

Discussion

Biologically foreign antiarrhythmic drugs, such as quinidine, procainamide, ajmaline, lidocaine, disopyramide, verapamil and β-adrenoblockers, have been commonly used, but care is necessary because their effective doses are close to the toxic doses, and the drugs or their metabolites might accumulate in the body for a long time. AAP is an intravital hexapeptide with antiarrhythmic ativity, obtained from atria.[1,3,4,12] This peptide has been

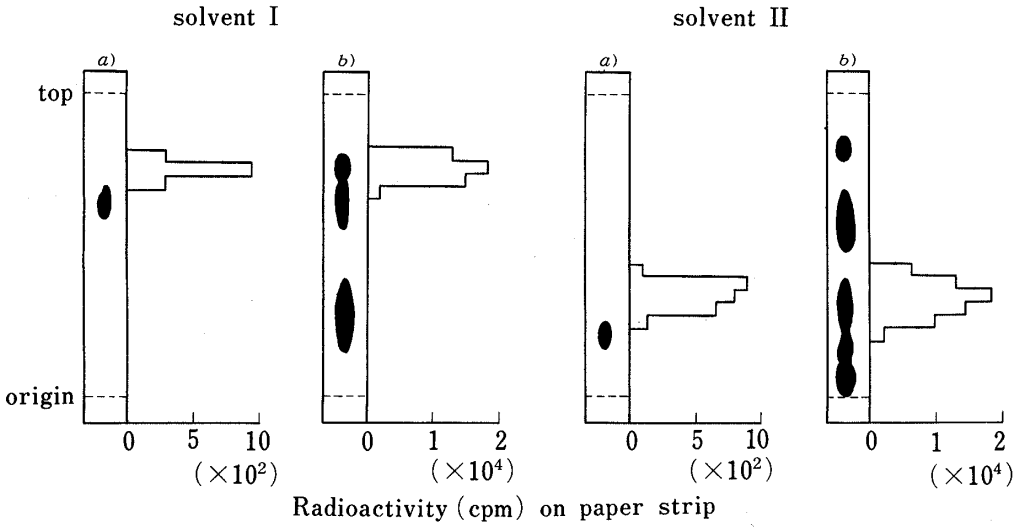

Fig. 8. Scintigrams obtained by Paper Chromatography of Serum Eluate S and Urine in Mice Administered with ^{14}C-AAP

a) Serum eluate S was obtained from blood of mice 60 min after oral administration of ^{14}C-AAP, as shown in Fig. 7. Staining, ninhydrin reagent (dark area).
b) Urine was obtained from mice within 60 min after intravenous injection of ^{14}C-AAP. Stiaining, KI-starch (dark area). Each sample was mixed with AAP (1 mg) as a carrier and applied to the paper. Solvens were the same as in Fig. 1.

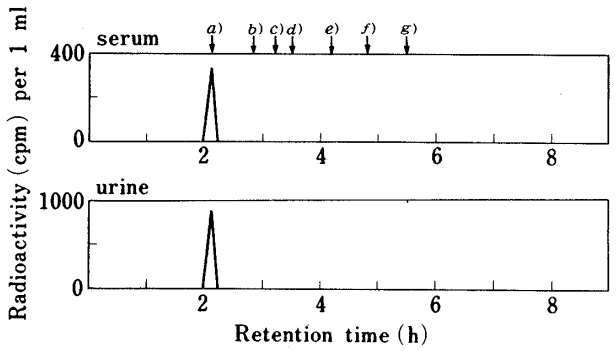

Fig. 9. Chromatograms obtained by Ion Exchange Chromatography of Serum and Urine in Mice Administered with ^{14}C-AAP

The column (Hitachi custom 2611, 0.9×55 cm) was eluted stepwise (pH 3.25 to pH 4.25 at 2 h 40 min, then pH 6.10 at 5 h 40 min)at 30 ml/h at 55°C. Fractions of 3 ml were collected. Serum was obtained from the blood of mice 60 min after oral administration of ^{14}C-AAP. Urine was obtained from mice within 60 min after intravenous injection of ^{14}C-AAP.
The retention times of authentic samples are indicated by arrows[4]: *a*) ^{14}C-AAP, *b*) AAP, *c*) acetamidino-Giy, *d*) Gly, *e*) Gly-Pro-Hyp-Gly, *f*) Gly-Pro-Hyp and *g*) Gly-Pro.

found to prolong the duration of electrical systole (QTc interval) in the electrocardiogram (lead II) without any effect on respiration rate or blood pressure.[1] Furthermore, even doses of 40—1000 times those (1—25 mg/kg, *i.v.*) showing the biological activity did not produce symptoms of acute toxicity in mice.[1] The present experiments were designed to relate these biological properties of AAP to its behavior *in vivo*.

AAP was newly synthesized by the solid phase method. In the hydrolysis of the synthetic peptide by APM, the slow release of Pro as well as Hyp may be explained by the known specificity of the enzyme.[13] An example of the cleavage of a peptide bond after Pro in the peptide sequence was also reported in an active center peptide of carboxypeptidase B by Plummer,[14] such cleavage may be a result of contamination of APM with endopeptidases.

The ^{14}C-AAP prepared should be an acceptable tracer of AAP because the labeled molecule is close to the native drug in molecular size (AAP,470; ^{14}C-AAP, 513), in polarity, in hydrophobicity (retention time in ion exchange chromatography: AAP, 2 h 40 min; ^{14}C-AAP, 2 h 6 min. *Rf* value in paper chromatography: AAP, 0.70; ^{14}C-AAP, 0.79 in solvent I: AAP, 0.26; ^{14}C-AAP, 0.37 in solvent II) and in antiarrhythmic effect as shown in Table I.

Intravenously injected ^{14}C-AAP was distributed rapidly into many organs, especially the kidney, but not into the brain, spinal cord, the contents of stomach and intestine or the fetus, and the drug was mostly lost rather rapidly, within 60 min. At 180 min after injection, 68% of

dose had been excreted in the urine. It is well-known that blood content is highest in the lung, followed by the liver, kidney, and myocardium in that order. Direct counting of these organs showed that the radioactivity was distributed in that order, except for the kidney. Although the distribution of radioactivity in the myocardium of the target organ was only 0.1% of the dose at even 1 min after injection, it is noteworthy that the radioactivity in the myocardium increased during 1 to 6 min after injection, since we reported previously that the actions of AAP on arrhythmias and on the duration of the QTc interval occurs at 1 to 10 min after injection (*i.v.*).[1] The distribution of AAP in the myocardium can be presumed to amount to 2 to 5 μg/g myocardium as calculated from the 0.1% distribution of ^{14}C-AAP in the myocardium when AAP (10—25 mg/kg, *i.v.*) prevented arrhythmias of mice *in vivo*.[1] This distribution of AAP in the myocardium is in accord with the levels of 0.1 to 20 μg/ml medium, at which AAP improved the rhythmicity of myocardial cells in culture.[3] The accumulation of radioactivity in the kidney was considered to be mainly due to radioactivity in the urine, but this requires confirmation. In mice intravenously injected with ^{14}C-AAP the unchanged molecular form of ^{14}C-AAP, was detected in the urine within 60 min after injection, indicating that this peptide is not decomposed in the body, except in the gastro-intestinal ducts, into which ^{14}C might be excreted through the bile duct.

In mice orally administered with ^{14}C-AAP a small amount of ^{14}C-AAP was absorbed from the gastro-intestinal ducts in the original form, and distributed to the myocardium at 40 to 120 min at 1/2.5 of the concentration at 1 to 6 min after intravenous injection. Therefore, comparatively continuous action of AAP may be obtainable by the administration of larger doses *p.o.* rather than the effective dose *i.v.*

These results are consistent with the observed biological effect, the virtual absence of side effects on heart functions and the very low toxicity of AAP.

References

1) Part XXII: S. Aonuma, Y. Kohama, T. Makino and K. Hattori, *Yakugaku Zasshi,* accepted.
2) A part of this work was presented at the 102nd Annual Meeting of the Pharmaceutical Society of Japan, Osaka, April, 1982.
3) S. Aonuma, Y. Kohama, K. Akai, Y. Komiyama, S. Nakajima, M. Wakabayashi and T. Makino, *Chem. Pharm. Bull.*, **28**, 3332 (1980).
4) S. Aonuma, Y. Kohama, T. Makino and Y. Fujisawa, *J. Pharm. Dyn.*, **5**, 40 (1982).
5) J. Scotcher, R. Lozier and A.B. Robinson, *J. Org. Chem.*, **35**, 3151 (1970).
6) B. Gutte and R.B. Merrifield, *J. Biol. Chem.*, **246**, 1922 (1971).
7) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).
8) M.J. Hunter and M.L. Ludwig, *J. Am. Chem. Soc.*, **84**, 3491 (1962).
9) C.H. Hirs, S.M. Moore and W.H. Stein, *J. Biol. Chem.*, **219**, 623 (1956).
10) P.U. Nwangwu, T.L. Holcslaw and S.J. Stohs, *Arch. Int. Pharmacodyn.*, **229**, 219 (1977).
11) D.T. Mahin and R.T. Lofberg, *Anal. Biochem.*, **16**, 500 (1966).
12) S. Aonuma, Y. Kohama, K. Akai and S. Iwasaki, *Chem. Pharm. Bull.*, **28**, 3340 (1980).
13) E.D. Wachsmuth, I. Fritze and G. Pfeiderer, *Biochemistry,* **5**, 175 (1966).
14) T.H. Plummer, JR., *J. Biol. Chem.*, **244**, 5246 (1969).