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# Microautoradiographic Distribution of $^{14}$C-Diltiazem in the Dog Kidney after Renal Arterial Injection

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The microscopic distribution of $^{14}$C-diltiazem in the dog kidney after renal arterial injection was studied by means of microautoradiographic technique. The highest silver grain density existed in the proximal and distal tubules, and the ascending loop of Henle. The definite accumulation of the radioactivity in the distal tubule and the ascending loop of Henle, which are involved in Na-transport, seems to be compatible with the observation that diltiazem affects directly on these segments, thus causing the diuretic action. The presence of radioactivity in the proximal tubule may also suggest that diltiazem has an influence on this tubule and increases the urinary volume.

**Keywords**——$^{14}$C-diltiazem; renal distribution; microautoradiography; diuretic action; dog

*d*-3-Acetoxy-*cis*-2,3-dihydro-5-[2-(dimethylamino)ethyl]-2-(*p*-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one hydrochloride (diltiazem) is a strong coronary vasodilator.[2)]

In addition, it is known that this compound increases the glomerular filtration rate and the urinary volume after renal arterial or systemic administration to anesthetized dogs.[3,4)] Diuretic action of diltiazem is also recognized in the clinical study and one of its mechanisms is assumed to be the inhibition of Na-reabsorption in the renal tubules.[5)]

In the present paper, the distribution of $^{14}$C-diltiazem in the dog kidney was examined by a microautoradiographic technique.

## Materials and Methods

**Labeled Compound**——The labeled diltiazem was synthesized with $^{14}$C in N methyl group as described earlier[6)] and its specific activity was 3.6 μCi/mg. Thin-layer and paper chromatography revealed one spot in which more than 98.0% of the radioactivity was located. Fig. 1 shows the chemical structure and the labeled position.

**Experimental Animals**——Six male mongrel dogs weighing 11.5 to 13.5 kg were used in the experiments. Three dogs (12.6, 13.0, 13.5 kg) were used for autoradiography and others (11.5, 11.6, 13.5 kg) were for tissue extraction studies.

**Autoradiography**——Three dogs were anethetized with sodium pentobarbital (30 mg/kg, *i.v.*), midline incision was made and $^{14}$C-diltiazem was injected in a dose of 1.5 mg/kg into the left renal artery over 15 sec. Since the maximal increase of the glomerular filtration rate occurs one minute after injection of diltiazem,[3)] the renal pedicle was ligated at this time and then the kidney was rapidly excised. It was cut into small blocks at about 5 mm in thickness as shown in Fig. 2. They were quickly frozen by immersion into isopentane

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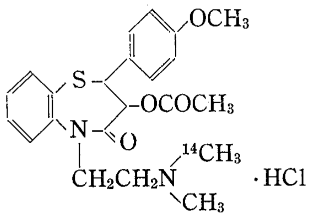

Fig. 1. Chemical Structure of $^{14}C$-Diltiazem

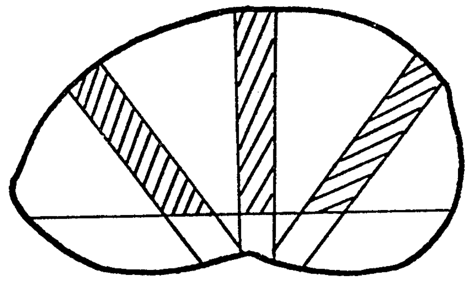

Fig. 2. The Kidney was cut into Small Blocks as shown above, and the Hatched Portions were used for sectioning

cooled with liquid nitrogen to about $-160°$. Thin 6 μm sections of the kidney were cut with a microtome of the Jung type (Yamato Kohki Co., Ltd.) in a cryostat at $-20°$. They were mounted on the slide painted thinly with egg-albumin glycerine, and freeze-dried overnight at $-20°$.

The photographic emulsion was applied by the wire loop method.[7,8] Nuclear emulsion (Sakura NR-M2) was diluted with 1.5 volumes of distilled water and warmed in a water bath at 40°. A thin film of the emulsion was obtained by dipping a wire loop into the emulsion. The loop film was air-dried and held firmly in contact with the specimen on the slide. Sections and films were stored in light-proofed boxes containing a desiccant and exposed for 4—8 weeks at 4°. They were developed with Konidol X for 2—8 min at 20°, stopped by 1.5% acetic acid, fixed with Konifix (Konishiroku Photo Industry Co., Ltd.), and stained with hematoxylin-eosin. In the control experiment sections were prepared with the kidney of a non-injected dog, and were autoradiographed as described above. The autoradiograms were assessed by visualizing the developed silver grains with the light microscope. Semiquantitation of $^{14}C$ distribution was made by counting the silver grains seen under the microscope over individual areas of the preparations. The result was expressed in five grades according to the number of grains per 100 $\mu m^2$; very high grain density (+++++, 41—50 grains/100 $\mu m^2$), high (++++, 31—40 grains/100 $\mu m^2$), moderate (+++, 21—30 grains/100 $\mu m^2$), low (++, 11—20 grains/100 $\mu m^2$), very low (+, 6—10 grains/100 $\mu m^2$) and background (−, 0—5 grains/100 $\mu m^2$).

Macroautoradiography was also carried out with the same kidney block by the method reported previously.[9,10]

**Tissue Extraction Studies**——Three dogs were injected renal-arterially with $^{14}C$-diltiazem in a dose of 1.5 mg/kg over 15 sec. They were sacrificed after one minute and kidneys were removed. Kidneys were homogenized with 5 volumes of saturated ammonium carbonate and were extracted with 2 volumes of ethyl acetate. Ethyl acetate extracts were chromatographed on thin-layer plates of silica gel $60F_{254}$ (Precoated, Merck) using benzene: dioxane: water: diethylamine (70: 17.5: 1: 7.5) and chloroform: methanol (100: 1). The radioactivity on the thin-layer plates was determined first by an Aloka thin-layer chromatogram scanner (TRM-1B) and secondly by an Aloka liquid scintilation spectrometer (LSC-652) after scraping the silica from the radioactive areas.

## Results

### Autoradiography

The macroautoradiographic study demonstrated that high concentration was found almost evenly in the cortex, while the inner medulla as well as blood vessels showed extremely low radioactivity. Detailed localization of radioactivity in the cortex, however, was not detected in the macroautoradiogram. Thus, the microautoradiographic examination was carried out.

Microautoradiographic examination (Fig. 3—5) revealed that very high grain density was found heterogeneously in the outer cortex. High grain density was seen in the medullary rays of the inner cortex and the outer zone of the medulla, though the inner zone of the

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medulla showed low grain density. In the lumen of the blood vessels and in the glomerulus the grain density was very low compared with other tissues. The semiquantitative distribution of the grains over individual renal structures is summarized in Table I. It was

Table I. Semiquantitative Distribution of the Silver Grains in Various Structures of the Dog Kidney One Minute after Renal Arterial Injection of $^{14}C$-Diltiazem

| Region of the kidney | | Grain density[a] |
|---|---|---|
| Outer cortex | Proximal tubule | +++++ |
| | Distal tubule | +++++ |
| | Glomerulus | + |
| Inner cortex (medullary ray) | Proximal tubule | +++~+++++ |
| | Distal tubule | +++ |
| Juxtamedullary region | Proximal tubule | +++ |
| | Distal tubule | +++ |
| | Glomerulus | + |
| | Blood vessel | + |
| Outer medulla | Henle's ascending | +++++ |
| | Collecting duct | ++ |
| | Capillary | + |
| Inner medulla | Collecting duct | ++ |
| Control tissue | | − |

a) Symbols: +++++ 41—50 grains/100 $\mu m^2$
++++ 31—40
+++ 21—30
++ 11—20
+ 6—10
− 0— 5

demonstrated from Fig. 3 and 5 that the largest amount of silver grains was found in the proximal and distal tubules and the ascending loop of Henle. The more detailed description on the distribution of radioactivity in this organ was as follows.

Fig. 3 shows the microautoradiograms in the outer cortex. As shown in this figure very high grain density was almost equally observed in the proximal and distal tubules; grain density was higher in the epithelial cells rather than the tubular lumen, but the accumulation in the nucleus was not observed. On the contrary, very low grain density existed in the glomerulus. Slightly higher grain density than the background was found in the interstitial tissue.

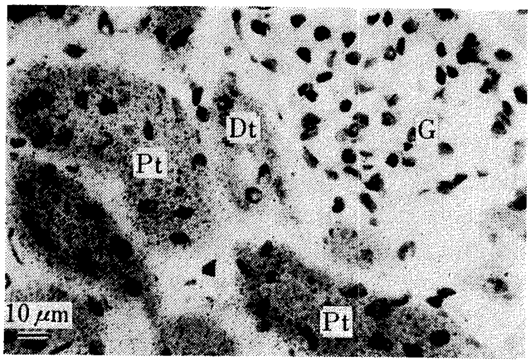


Fig. 3. Microautoradiograms of the Dog Kidney (Outer Cortex) after Renal Arterial Injection of $^{14}C$-Diltiazem

High grain density is observed in the outer cortex. The proximal and distal tubules show very high grain density, though the glomerulus has very low grain density (×400).

Abbreviations: Dt; distal tubule, G; glomerulus, Pt; proximal tubule.

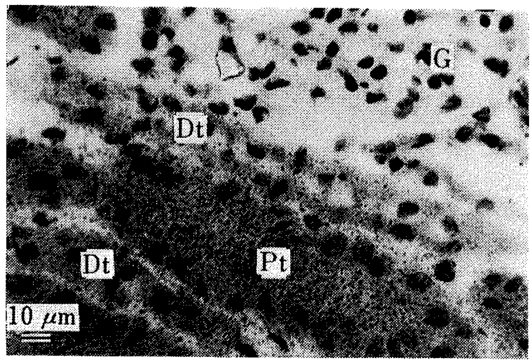


Fig. 4. Microautoradiogram of the Dog Kidney (Inner Cortex) after Renal Arterial Injection of $^{14}C$-Diltiazem

Silver grains are prominent in the medullary ray especially in the proximal tubule (×400).

Abbreviations: Dt; distal tubule, G; glomerulus, Pt; proximal tubule.

Fig. 4 shows the medullary ray and the glomerulus in the inner cortex. High grain density was found in the medullary ray, particularly in the straight portion of the proximal tubule, whereas glomerulus had very low grain density, as observed in the outer cortex.

In the juxtamedullary region the grain density was lower than in the outer cortex. But the distribution pattern was the same as seen in the outer zone; high in the proximal and distal tubules, and low in the glomerulus. Radioactivity in the wall of the blood vessels was moderately observed as seen in the outer cortex, while radioactivity in the blood was scarcely detected.

Microautoradiogram in the outer zone of the medulla is shown in Fig. 5. Very high grain density was observed in the ascending loop of Henle. The rete mirabile, bundle of blood capillaries, had very low grain density.

In the inner zone of the medulla low grain density was observed in the collecting duct, though the radioactivity was detected moderately in the lumen of ureter.

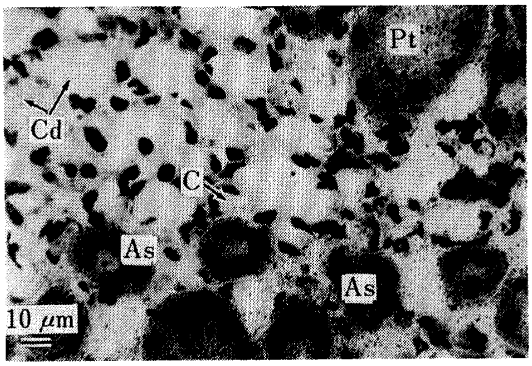


Fig. 5. Microautoradiogram of the Dog Kidney (Outer Medulla) after Renal Arterial Injection of $^{14}$C-Diltiazem

High accumulation of grains are seen in the ascending loop of Henle, though low in the collecting duct and the capillary (×400).
Abbreviations: As; ascending loop of Henle, C; capillary, Cd; collecting duct, Pt; proximal tubule.

**Tissue Extraction Studies**

Approximately 95% of the total radioactivity in the kidney was recovered in the ethyl acetate phase. About 3% of the radioactivity remained in the water phase and in the pellet. At thin-layer chromatography of the organic phase, 97% of the radioactivity moved in a spot corresponding to unchanged diltiazem. Less than 3% of the activity was found in a spot corresponding to deacetylated compound.

## Discussion

The results of macroautoradiograms roughly revealed the distribution of radioactivity in the dog kidney after injection of $^{14}$C-diltiazem. However, detailed localization could not be found because of its low resolution. To get more detailed information of the renal distribution, microautoradiographic study was carried out. In the microautoradiography the radioactive material in the tissue can be detected microscopically as the silver grains in the photographic emulsion applied to the specimen. In experiments with diffusible substances[11,12,13] it is important to avoid translocation of the radioactive materials in the tissue during both the histological procedure and the application of the photographic emulsion. For this reason, non-fixed and non-embedded freeze-dried sections were prepared in the cryostat at $-20°$ and the wire loop method[7,8] was used to get a good contact of dry films to freeze-dried sections.

In order to determine whether the radioactivity in the kidney did indeed represent diltiazem itself, the kidneys were removed from the dogs treated with $^{14}$C-diltiazem and assayed with thin-layer chromatography. Thin-layer chromatography revealed that more than 92% of the radioactivity in the kidney represented unmetabolized $^{14}$C-diltiazem.

As demonstrated in the present experiments, high grain density was observed in the proximal and distal tubules and the ascending loop of Henle which are related with Na-reab-

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sorption. Tsuchiya, *et al.*[5] reported that the increase of urinary volume found clinically after the administration of diltiazem may be responsible for the increase of Na excretion rather than the acceralation of the glomerular filtration rate, and that the natriuretic action of diltiazem may be ascribable to the inhibition of Na-reabsorption in the ascending loop of Henle and the distal tubule. Yamaguchi, *et al*[3] also suggested that the natriuretic action of diltiazem is not only due to the changes in renal hemodymanics but also may be ascribed to the direct inhibition of Na-reabsorption in the distal tubule. Consequently, the high uptake of the radioactivity into the distal tubule and the ascending loop of Henle is assumed to be related to the diuretic action of diltiazem, and may support the above observations that one of its mechanisms is the inhibition of Na-reabsorption in these segments. Deetjen[14] demonstrated by micropuncture techniques that the functional sites of diuretic action of frosemide, which is a typical natriuretic agent, mainly localized in the ascending loop of Henle and the distal tubule and probably in the proximal tubule. Though the effect of diltiazem in the proximal tubule is not demonstrated, the uptake of radioactivity into the tubule may also suggest that diltiazem causes the diuretic action by affecting on this segment.

The microautoradiogram indicated that the radioactivity was highly distributed in the tubular cells, while it was low in the tubular lumen and slightely detected in the collecting duct. Thus, it is inferred that diltiazem may be reabsorbed from the tubular lumen and retained in these tubules.

Abe, *et al.*[4] investigated the effect of diltiazem on the renal blood flow by the radioactive microsphere technique. They reported that the increase of renal blood flow produced by renal arterial administration of diltiazem in anesthetized dogs was more prominent in the inner zone of the cortex than the ourter zone. In the present study, however, the grain density in the wall of the blood vessels did not show apparent difference in each zone of the cortex.

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