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Distribution of acotiamide, an orally active acetylcholinesterase inhibitor, into the *myenteric plexus* of rat and dog stomachs

Kazuyoshi Yoshii [a,*], Takashi Yamaguchi [a], Masamichi Hirayama [a], Ryoko Toda [a], Toshiko Kinomoto [a], Yoshihiro Kawabata [a], Kan Chiba [b]

[a] Toxicology & Pharmacokinetics Research, Central Research Laboratories, Zeria Pharmaceutical Co., Ltd., Saitama, Japan
[b] Laboratory of Pharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan

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ABSTRACT

Aims: Acotiamide is the first-in-class drug for the treatment of functional dyspepsia. Although pharmacological and therapeutic actions of acotiamide are thought to be derived from its inhibitory effects on acetylcholinesterase (AChE), whether the concentration of acotiamide at the site of action is sufficient to inhibit AChE remains unclear. Since major site of acotiamide action is thought to be the cholinergic nerve terminals in gastric *myenteric plexus*, we studied the distribution of [^{14}C]acotiamide into gastric *myenteric plexus*.

Main methods: Distribution of [^{14}C]acotiamide was evaluated using macro- and micro-autoradiography in rats and dogs.

Key findings: The results of macro-autoradiography showed the concentration of radioactivity was 27.9 μM in rat stomach, which was 12 times higher than IC_{50} of acotiamide for rat AChE. Being different from rats, the distribution of radioactivity in the muscular layer was distinguishable from that in the mucosal layer in dog stomach. The concentration of radioactivity in the muscular layer of dog stomach (1.41 μM) was approximately two-times lower than those in the mucosal layer, however, it was approximately 1.2 times higher than IC_{50} of acotiamide for dog AChE. The results of micro-autoradiography also showed the radioactivity distributed homogenously in the muscular layer of rat stomach, suggesting the concentration of radioactivity around the ganglion of *myenteric plexus* is similar to that in the muscular layer of stomach.

Significance: These findings suggest acotiamide distributes to the *myenteric plexus* of stomach, a putative site of acotiamide action, with adequate concentrations to inhibit AChE, in both of rat and dog stomachs.

1. Introduction

Functional dyspepsia (FD) is a common gastrointestinal disorder defined as symptom-based conditions in the absence of organic disease [1]. Symptoms are categorized as postprandial distress and epigastric pain syndromes, which are associated with impaired gastric accommodation and emptying [2,3]. Gastric accommodation and emptying are induced by coordinating motility of gastric fundus, body and antrum, which are regulated by complex nervous systems including cholinergic neurons projected from dorsal motor nucleus of the vagus to the stomach [4,5].

Acotiamide is the first-in-class drug for the treatment of FD [6]. Although some clinical studies have suggested that proton pump inhibitors (*e.g.*, omeprazole) and prokinetics (*e.g.*, itopride or mosapride) are effective, no product except for acotiamide has gotten marketing approval for the treatment of patients with FD [6]. Clinical studies have shown that acotiamide enhances the gastric accommodation reflex and gastric emptying rate [7] and improves meal-related symptoms such as postprandial fullness, upper abdominal bloating and early satiation in patients of FD [8]. These pharmacological and therapeutic effects of acotiamide are thought to be derived from its inhibitory effects on acetylcholinesterase (AChE) which results in the potentiation cholinergic neurons [9,10]. In fact, an *in vitro* animal experiment showed that acotiamide enhanced acetylcholine (ACh)-induced but not carbachol (not hydrolyzed by AChE)-induced contraction of isolated gastric antrum strips of guinea pig [10]. In addition, an *in vivo* animal experiment showed that acotiamide enhanced gastric body contractions induced by electrical stimulation of the vagus nerve in rats, which were completely abolished by classical antagonist of ACh receptors [11].

Acotiamide inhibits AChE with half maximal inhibitory concentrations (IC_{50}) of 3.0, 2.3 and 1.2 μM for human recombinant, and rat and canine gastric AChEs, respectively [10,11]. These values are much larger

* Corresponding author at: 2512-1, Numagami, Oshikiri, Kumagaya-shi, Saitama 360-0111, Japan.
E-mail address: kazuyoshi-yoshii@zeria.co.jp (K. Yoshii).

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than those of classical inhibitors of AChE, such as neostigmine, however, acotiamide is concentrated into the stomach tissue by carrier-mediated uptake processes, which may account for the selective action of acotiamide for gastric smooth muscle but not for skeletal muscle and central nervous system in rats [12].

Myenteric plexus is a mesh-like system of neurons which provide major motor innervation to the gastrointestinal muscles [13]. Therefore, main target of acotiamide action is thought to be AChE localized around the cholinergic nerve terminals in the *myenteric plexus* which is located in the muscular layer of the stomach. However, whether sufficient concentrations of acotiamide are attained in the *myenteric plexus* of the stomach to inhibit AChE, has not been confirmed in any species yet, although the total concentrations of acotiamide in the homogenate of rat stomach after *in vivo* administration were reported to be higher than *in vitro* IC_{50} value of acotiamide estimated from rat gastric AChE [11].

The aim of the present study was to examine the distribution of acotiamide into the *myenteric plexus* of the stomach after [^{14}C] acotiamide dose enough to exhibit pharmacological action, using macro- and micro-autoradiographs, and to estimate whether the sufficient concentration of acotiamide is attained in the *myenteric plexus* of the stomach, a putative site of acotiamide action, to inhibit AChE in rat and dog stomachs.

2. Materials and methods

2.1. Chemicals

N-[2-[bis(1-methylethyl)amino]ethyl]-2-[(2-hydroxy-4,5-dimethoxybenzoyl)amino]thiazole-4-carboxamide monohydrochloride trihydrate (acotiamide hydrochloride, Z-338/YM443, Fig. 1) was synthesized in the central research laboratories of Zeria Pharmaceutical Co., Ltd. (Saitama, Japan). [^{14}C]Acotiamide (2.26 GBq/mmol) was synthesized by GE Healthcare UK Ltd. (Buckinghamshire, England). All other chemicals were of reagent grade.

2.2. Animals

Male Sprague–Dawley rats aged six to seven weeks were obtained from Charles River Japan, Inc. (Kanagawa, Japan) and housed under standard controlled environmental conditions at 23 ± 3 °C and 55 ± 20% humidity with a 12-h light/dark cycle, and food (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan or CE-2; CLEA Japan Inc., Tokyo, Japan) and water available *ad libitum*. Rats were allowed to acclimate to laboratory conditions for at least one week prior to experiments.

Male beagle dogs were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan) or the Institute for Animal Reproduction (Ibaraki, Japan) and housed individually in experimental cages where they were acclimated for at least 12 days before entry to the study. Laboratory chow (NVE-10, Nihon Pet Food K.K., Tokyo, Japan; or DS-A, Oriental Yeast Co., Ltd.) was provided once daily and water was given *ad libitum*. Animals were housed under standard controlled environmental conditions at 22 ± 3 °C and 50 ± 20% humidity with a 12-h light/dark cycle.

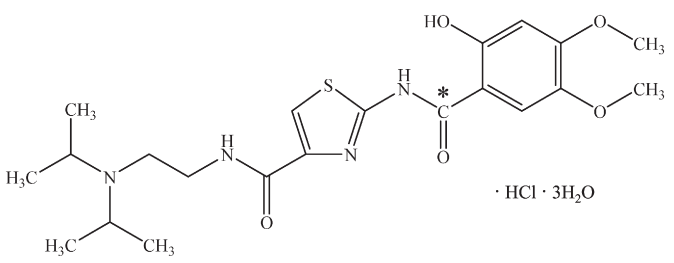

Fig. 1. Structure of acotiamide hydrochloride.

All animal experiments were approved by the Animal Care and Use Committee of the Central Research Laboratories of Zeria Pharmaceutical Co., Ltd., Animal Ethical Committee, Tsukuba Laboratories, Nemoto Science Co., Ltd. (Ibaraki, Japan) and the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd. (Wakayama, Japan).

2.3. AChE staining of rat stomach

Rats were exsanguinated *via* the abdominal aorta under anesthesia with diethyl ether and the stomach was excised, washed with saline, ligated at the pylorus, filled with OCT compound through the cardia, ligated at the cardia. Stomach was embedded with OCT compound and frozen in a bath of isopentane and dry ice. Embedded stomach was cut into 6-μm-thick sections using a cryomacrocut CM1900, and immersed in 3 mM copper sulfate/0.05 mM potassium ferricyanide solution containing acetylthiocholine iodide at 37 °C for 90 min. After rinsing in distilled water, sections were immersed in Mayer's hematoxylin for 10 min and then washed with running tap water, dehydrated in ethyl alcohol, and immersed in xylene.

2.4. AChE staining of dog stomach

Dogs were euthanized by intravenous administration of sodium pentobarbital and supersaturated potassium chloride solutions. Stomach was immediately excised, and divided into 2 pieces along the greater curvature. The pieces were rinsed with physiological saline and divided into approximately 1.5 cm × 1.5 cm sections and frozen in liquid nitrogen. Sections were embedded with an OCT compound and frozen in hexane and dry ice. The frozen serial sections were prepared as 8-μm sections using a cryomacrocut CM1900. The sections were immersed as described in AChE staining of stomachs of rats.

2.5. Macro-autoradiography of rat stomach

As any pharmacological effects haven't been reported after oral administration of acotiamide to rats although acotiamide is orally administered for the treatment of FD, rats were subcutaneously administered with [^{14}C]acotiamide (30 mg and 20.6 MBq/kg). These dose and route of acotiamide administration were reported to increase the gastric motility index significantly until 90 min after the administration to rats [11]. Rats were exsanguinated *via* the abdominal aorta under anesthesia with diethyl ether, and the stomach was excised at 30 min after the administration of acotiamide. The excised stomach was washed with saline, ligated at the pylorus, filled with 2% CMC-Na through the cardia, ligated at the cardia, embedded with 2% CMC-Na and frozen in a hexane/dry ice bath. The embedded stomach was cut into 40-μm-thick sections using a cryomicrotome CM3600 (Leica Microsystems GmbH, Wetzlar, Germany). Sections were exposed by contact with an Imaging-Plate BAS-III2040 (Fuji Photo Film, Tokyo, Japan) in a shield box for 16 h. After exposure, the Imaging-Plate was analyzed with a Bio-Imaging Analyzer BAS-2000 (Fuji Photo Film, Tokyo, Japan) to obtain macro-autoradioluminograms and measure radioactivities in the tissues. The concentration of the radioactivity in the tissue was quantified by the absolute calibration method [14,15,16,17]. The calibration curve through the origin ($Y = aX$) was prepared based on the mean value of the intensity of luminescence (PSL-BG/mm^2) of the blood simultaneously collected when stomach was removed and blood concentration of acotiamide determined by a liquid scintillation analyzer LSC-6100 (Aloka, Tokyo, Japan). The background (BG) value was calculated as the mean value of the intensity of luminescence in three positions on the rim of the section. The lower limit of quantification was defined as twice the BG value. Tissue concentration of acotiamide (mean ± standard deviation) was expressed as μM based on the assumption that tissue specific gravity equals 1 mL/g.

2.6. Macro-autoradiography of dog stomach

Dogs were anesthetized with medetomidine and isoflurane, and subjected to abdominal incision. A catheter was inserted into the duodenum from an incision and [^{14}C]acotiamide (30 mg and 1 MBq/kg) was administered. These dose and route of acotiamide administration were reported to increase the gastric motility index significantly at least until 30 min after the administration to dogs [18]. Dogs were euthanized by intravenous administration of 64.8 mg/mL sodium pentobarbital solution (2 mL/kg) at 30 min after dosing. The cardia of the stomach was tied with a silk suture and the stomach was promptly enucleated. The excised stomach was washed with saline and ligated at the pylorus. The stomach was embedded with 5% CMC-Na and frozen in a bath of hexane and dry ice. The embedded stomach was cut into 30-μm-thick sections using a cryomicrotome CM3600, which were exposed by contact with an Imaging-Plate BAS-MS2040 in a shield box for 16 h. After exposure, the Imaging Plate was analyzed with the Bio-Imaging Analyzer BAS-2500 (Fuji Photo Film, Tokyo, Japan) to obtain macro-autoradioluminograms and measure radioactivities in the tissues. The concentration of the radioactivity in the tissue was quantified by the absolute calibration method as described in macro-autoradiography of rat stomach. Tissue concentration of acotiamide (mean ± standard deviation) was expressed as μM based on the assumption that tissue specific gravity equals 1 mL/g.

2.7. Nissl staining and micro-autoradiography of rat stomach

Rats were treated with [^{14}C]acotiamide and the stomach was excised as described in macro-autoradiography of rat stomach. Excised stomach was washed with saline, immersed in 10% formalin, and then placed in gum sucrose solution. Once complete submersion was confirmed, stomach was ligated at the pylorus, filled with OCT compound through the cardia, ligated at the cardia, embedded with an OCT compound and frozen in liquid nitrogen. Serial sections of 6-μm-thick were obtained with a cryomacrocut CM1900 (Leica Microsystems GmbH) and attached onto MAS-coated glass slides (Matsunami Trading Co., Ltd., Osaka, Japan) covered with emulsion for the micro-autography and without emulsion for Nissl staining. For Nissl staining, sections were dehydrated in ethanol, rehydrated in distilled water and submerged in 0.1% cresyl violet solution for approximately 30 min until the desired depth of staining was achieved. Sections were dehydrated in ethanol, immersed in xylene, and covered with a coverslip. For the micro-autoradiography, sections were set in a slide glass case and dried completely, then exposed in a refrigerator at 4 °C for 2 weeks. Sections were developed, subjected to hematoxylin and eosin staining, and finally covered with a coverslip.

3. Results and discussion

Although the localization of AChE in the muscular layer of the stomach has been reported for humans [19], little information is available on the experimental animals. Therefore, we studied the localization of AChE in the muscular layer of rat and dog stomachs using AChE staining. As shown in the Fig. 2, AChE-activity was detected around the nerve cells in the muscular layer of the rat stomach, which were located between the circular and longitudinal muscles, indicating that the nerve cells are components of the *myenteric plexus*. Similarly, AChE-activity was also found in the ganglia of the *myenteric plexus* of the dog stomach (Fig. 3). These findings are consistent with those of humans [19], and suggest that major target of acotiamide action in rats and dogs is AChE in the ganglia of *myenteric plexus* located in the muscular layer of the stomach.

Macro-autoradiograms of rat and dog stomachs after the administration of [^{14}C]acotiamide are shown in Figs. 4 and 5, respectively. The autoradiograms of rat and dog stomachs showed no marked difference of the radioactivity between the stomach sections. The mean concentration of acotiamide in the stomach of rats estimated from the radioactivity was 27.9 ± 10.4 μM (n = 3), which was 12 times higher than IC_{50} of acotiamide (2.3 μM) estimated from an *in vitro* experiment using rat gastric AChE [11]. Although mucosal and muscular layers were not distinguishable in the rat stomach (Fig. 4), they were distinguished in the dog stomach (Fig.5). The mean concentration of radioactivity in the muscular layer of the dog stomach (1.41 ± 0.18 μM, n = 3) was approximately two-times lower than that of mucosal layer (3.24 ± 0.66 μM, n = 3), however, it was approximately 1.2 times higher than IC_{50} of acotiamide (1.2 μM) estimated from an *in vitro* experiment using dog gastric AChE [10].

Nissl stained and micro-autoradiographic images of the muscular layer in the rat stomach are shown in Fig. 6A and B, respectively. As shown in the Fig.6A, Nissl stain clearly visualized nerve cells of *myenteric plexus* in the muscular layers of rat stomach. Micro-

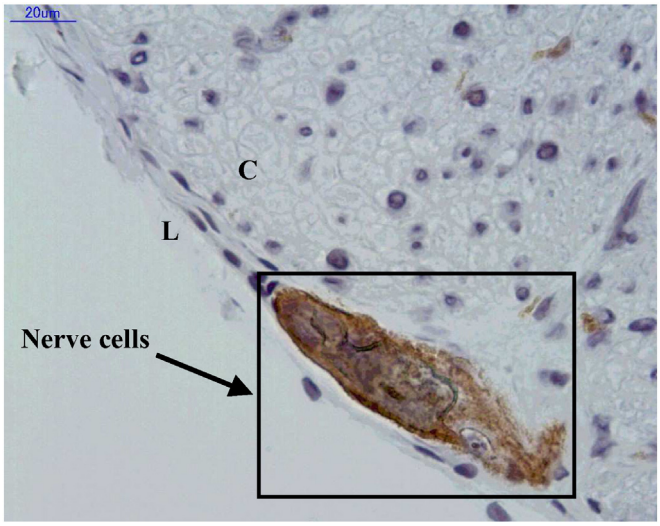

Fig. 2. Photomicrograph of AChE activity staining for the muscular layer of rat stomach. The stomach was embedded with OCT compound. Embedded stomach was cut into 6-μm-thick sections and immersed in 3 mM copper sulfate/0.05 mM potassium ferricyanide solution containing acetylthiocholine iodide at 37 °C for 90 min. Then, sections were immersed in Mayer's hematoxylin, dehydrated in ethyl alcohol, and immersed in xylene. Scale bar represents 20 μm. Arrow in photomicrograph indicates nerve cells, and brownish site indicates AChE activity staining located between the circular (C) and longitudinal (L) muscles.

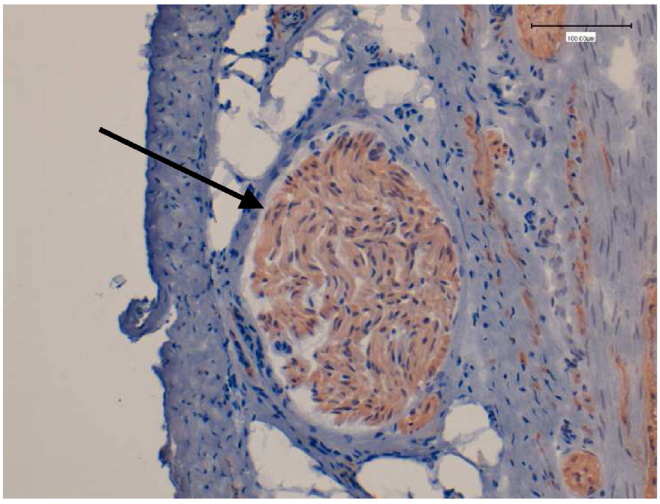

Fig. 3. Photomicrograph of AChE activity staining for the muscular layer of dog stomach. The stomach was embedded with OCT compound. Embedded stomach was cut into 8-μm sections and immersed as described in AChE staining of rat stomach. Scale bar represents 100 μm. Arrow in photomicrograph indicates the ganglia, and brownish site indicates AChE activity staining.

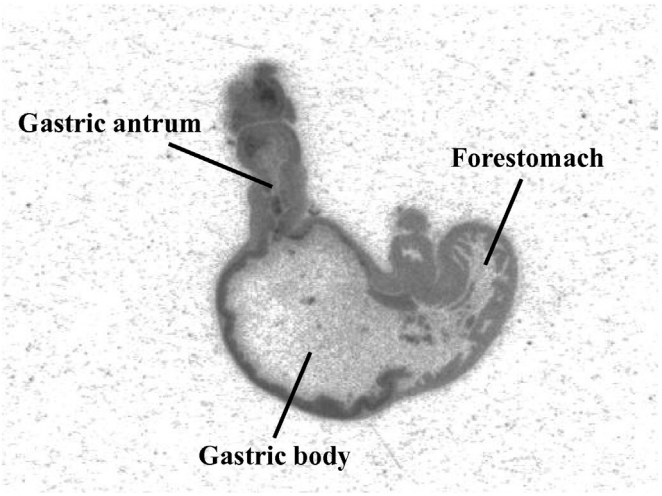

Fig. 4. Macro-autoradiogram of rat stomach. Thirty mg/kg of [^{14}C]acotiamide (20.6 MBq/kg) were administered subcutaneously to rats and the stomach was excised at 30 min after the administration. The excised stomach was embedded with 2% CMC-Na, sliced into 40-μm-thick sections, exposed to imaging plate for 16 h and analyzed with imaging analyzer.

autoradiogram also showed that the radioactivity distributed homogenously in the muscular layer (the radioactivity was detected as black-silver grains) and distribution of the radioactivity was not apparently different between the surrounding of nerve cells and other regions of muscular layer of rat stomach (Fig. 6B). The results suggest that the concentration of radioactivity around the ganglion of the *myenteric plexus* is similar to that in the muscular layer of the rat stomach. However, in the case of rats, the result of the macro-autoradiography showed that the muscular layer could not be distinguished from mucosal layer (Fig. 4) probably due to the thinness of the rat gastric wall. Thus, it may be impossible to estimate the concentrations of radioactivity directly from the macro-autoradiography. Nonetheless, assuming the difference in the concentrations of acotiamide between muscular and mucosal layers found in dog stomach is similar to that in rat stomach, the concentrations of radioactivity in the muscular layer of rat stomach are estimated to be 8.5 μM, which was still much higher than IC_{50} of acotiamide (2.3 μM) estimated from an *in vitro* experiment using rat gastric AChE [11]. On the other hand, in the case of dogs, the concentrations of radioactivity in the muscular layer could be directly estimated from macro-autoradiography, therefore the concentrations of radioactivity around the ganglion of the *myenteric plexus* would be identical to those estimated from macro-autoradiography (1.41 μM). The concentration was higher than IC_{50} of acotiamide for dog gastric AChE (1.2 μM) [10], as described above. These findings suggest that adequate amount of acotiamide could distribute into the ganglion of the *myenteric plexus* of the stomach to inhibit AChE in both of the rat and dog stomachs.

Finally, it should be notified that radioactivity is not always identical to acotiamide but can be metabolites of acotiamide or the mixture of acotiamide and its metabolites. Although we do not have direct evidence that radioactivity in the stomach mainly represents unchanged acotiamide, the concentration of acotiamide in the homogenate of rat stomach determined by high performance liquid chromatography reported previously [11] is not so different from the concentration of the radioactivity in the rat stomach found in the present study. Thus, the radioactivity found in the rat stomach is considered to be mainly unchanged acotiamde. In the case of dogs, the concentration of acotiamide in the homogenate of stomach has not been reported. However, plasma concentration-time profiles of acotiamide and radioactive acotiamide after the administration of the same dose of acotiamide as the present study to dogs indicate that plasma concentration of unchanged acotiamide at 30 min (equal to the sampling time of dog stomach in the present study) after the oral administration is almost identical to that of radioactivity at 30 min after the oral administration of the same dose of [^{14}C]acotiamide to dogs (Yoshii et al., unpublished observation). The findings suggest that radioactivity mainly represents unchanged acotiamide at least in plasma at 30 min after the administration of [^{14}C]acotiamide. The findings also suggest that acotiamide is not extensively metabolized at 30 min and unchanged acotiamide appears to predominantly distribute into the stomach at 30 min after the oral administration to dogs. Thus, the radioactivity found in the dog stomach is also considered to be mainly unchanged acotiamide.

4. Conclusions

The results of the present study showed that the radioactivity distributes into the *myenteric plexus*, a putative site of acotiamide action,

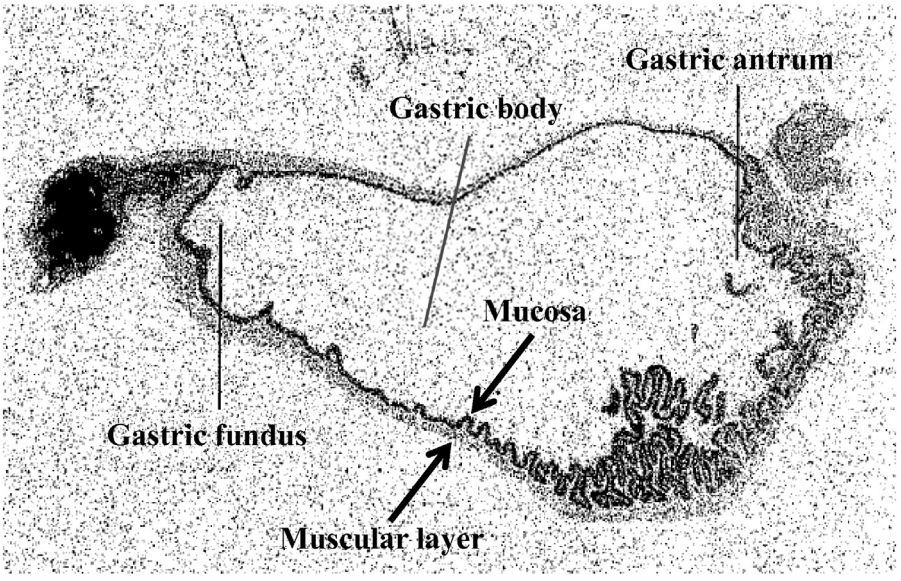

Fig. 5. Macro-autoradiogram of dog stomach. Thirty mg/kg of [^{14}C]acotiamide (1 MBq/kg) were administered intra-duodenally to dogs and the stomach was excised at 30 min after the administration. The stomach was embedded with 5% CMC-Na, sliced into 30-μm-thick sections, exposed to imaging plate for 16 h and analyzed with imaging analyzer.

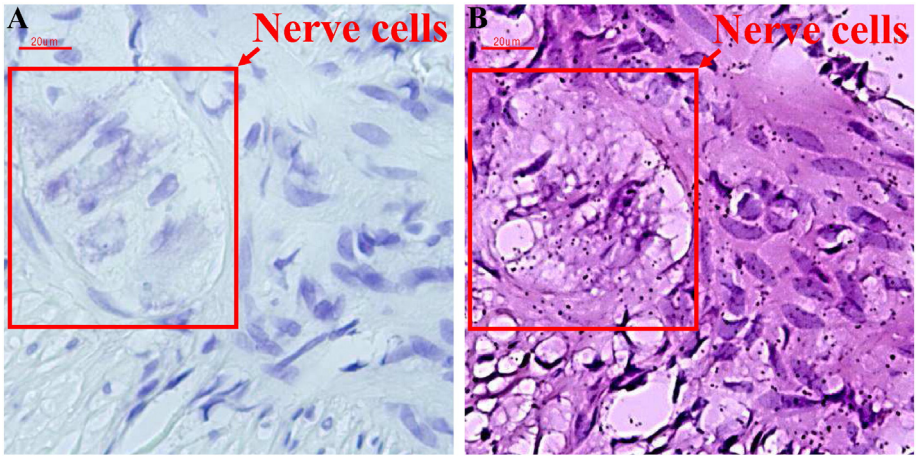

Fig. 6. Nissl-stained photomicrograph (A) and micro-autoradiogram (B) of the rat stomach. Thirty mg/kg of [^{14}C]acotiamide (20.6 MBq/kg) were administered subcutaneously to rats and the stomach was excised at 30 min after the administration. The stomach was embedded with OCT compound. Embedded stomach was cut into 6-μm-thick sections. Serial sections were attached onto glass slides covered with emulsion for the micro-autography and without emulsion for Nissl staining. For Nissl staining, sections were dehydrated in ethanol, rehydrated in distilled water and submerged in 0.1% cresyl violet solution for approximately 30 min. Sections were dehydrated in ethanol, immersed in xylene, and covered with a coverslip. For the micro-autoradiography, sections were dried completely, then exposed in a refrigerator at 4 °C for 2 weeks. Sections were developed, subjected to hematoxylin and eosin staining, and finally covered with a coverslip. Scale bars represent 20 μm. The red arrows indicate nerve cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in the muscular layer of the rat and dog stomachs, and that the concentrations of radioactivity around the *myenteric plexus* are estimated to be adequate to inhibit AChE in rats and dogs. Since the dose of acotiamide used in the present study was enough to exhibit pharmacological action in rats and dogs [11,18], the present findings suggest that inhibition of gastric AChE is a major mechanism of action responsible for the pharmacological effects of acotiamide on the gastrointestinal tract of rats and dogs, and may be for the therapeutic effects on the symptoms of patients with FD.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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