HISTOCHEMICAL DEMONSTRATION OF CHINOFORM

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Chinoform (7-iodo-5-chloro-8-hydroxyquinoline) which is a derivative of oxin (8-hydroxyquinoline) can chelate with some metals as well as oxin and other derivatives. Applying this principle, the techniques of histochemical demonstration for chinoform, which is probably the causal agent of subacute myelo-optico-neuropathy (SMON), were devised.

Copper, iron and silver were used as substances to be chelated with chinoform and they were demonstrated histochemically. In using these techniques, it was necessary to perform these chemical reactions in Tween 80 solution.

The possibilities of these techniques were examined using various kinds of materials.

Insoluble chinoform hydrolyzed from chinoform glucuronide as a substrate by β -glucuronidase activity deposited *in situ* positively and was demonstrated histochemically. In addition, the devised techniques were tested and comparisons were made with the autoradiographic findings in the tissue sections of rabbits to which ^{131}I -chinoform was administered.

It has gradually been clarified by many investigations in Japan that a causal agent of subacute myelo-optico-neuropathy (SMON) could be a drug of antiamebic compounds called chinoform. If chinoform could be histochemically detected in organs or tissues of the SMON patient, it would be one proof that SMON was probably caused by chinoform. Moreover, it should be possible to examine the distribution of chinoform in tissue cells.

For this purpose, attempts should be made to find a technique for histochemical demonstration of chinoform *in situ*.

Chinoform, which is a derivative of oxin, chelates with some metals as well as oxin and other derivatives, and the compounds should be stable in Tween 80 solution. In this solution, combining a metal with chinoform in a tissue can be used as a histochemical technique for demonstration of chinoform.

In this study, the histochemical techniques using the metal chelate formation mechanism were devised for demonstrating chinoform and the possibility of their presence was examined by using some kinds of materials for microscopic visualization.

First, a deposition of chinoform in a tissue cell was newly applied in the liver tissue of a rabbit in which β -glucuronidase was abundantly present. Namely, by using a soluble chinoform glucuronide as the substrate, the β -glucuronidase activity

worked on it to deposit the insoluble chionoform in the cells of tissue sections by its hydrolysis and then the chionoform was histochemically demonstrated to be present by using the chelate formation mechanism.

Secondly, for confirmation of its distribution in tissue cells, ^{131}I -chionoform was administered perorally to rabbits and then the autoradiography was tried, then compared with the findings of the histochemical demonstration.

Thirdly, the possibility of the techniques was investigated using various kinds of tissues of rabbits poisoned by chionoform.

Lastly, the techniques for histochemical demonstration of chionoform were applied to the tissue of the SMON patients. In this paper, it was proved that these techniques were effective for demonstrating the presence of chionoform in organs and tissues.

MATERIALS AND METHODS

A) *Materials: Tissue and tissue preparation*

a) **Preparation of sections in which chionoform was deposited enzyme-histochemically by β -glucuronidase.**

10 mg chionoform glucuronide which was synthesized by Tamura *et al.* (10) was first dissolved at 40°C in 10 ml distilled water containing two drops of 0.1 N NaOH which made it easier to dissolve. After cooling at 37°C, this solution was adjusted at pH7.0 with two drops of 0.1 N HCl and then was buffered at pH5.2 with the equal volume of acetic buffer. The fresh cryostat liver sections of normal rabbit were incubated in this incubation mixture at 37°C for two hours. Insoluble chionoform was released from the substrate by β -glucuronidase, deposited *in situ*, and then the chionoform was histochemically demonstrated by using the chelate formation mechanism.

b) **Preparation of tissue sections of rabbits which were administered ^{131}I -chionoform.**

Rabbits weighing from 1,100 g to 1,300 g were utilized as the experimental animals. These animals were administered perorally 23 mg ^{131}I -chionoform (2.9 $\mu\text{-Ci}$) per 100 g of body weight, and killed at the time intervals of 1, 2 and 4 hours following the treatment. Liver, kidney, ischiadic nerve, and lumbar region of spinal cord were removed and fixed with 10 per cent formalin.

Using these tissues, the autoradiographic procedure was made with NR-M2 emulsion (Sakura) following the dipping method. Besides using the serial sections of the same tissue blocks the histochemical demonstration for chionoform was tried by devised techniques and the findings were compared with that of the autoradiograph.

c) **Preparation of tissue sections in rabbits to which chionoform was administered.**

A rabbit weighing 4,000 g was chosen from several animals treated with emaform which contained 95 per cent chionoform and CMC (carboxy methyl cellulose). 40 mg emaform per one kilogram of body weight was given at the beginning and the total dose of 19.1 g was given per one kilogram of body weight. The animal was killed 85 days after the beginning of the administration of the drug. The tissues were removed from the body and the paraffin sections were

routinely prepared. The histochemical demonstration for chinoform was tried in the liver tissue section.

Moreover, two experimental animals weighing 3,700 g (No. 22) and 4,400 g (No. 34) were utilized for the examination of distribution of administered chinoform in various organs. These animals were daily given 1 per cent emulsion of emaform.

One rabbit (No. 22) was given the dose of 40 mg emaform per one kilogram of body weight to begin with and the dose was gradually increased. The total dose given was 12.3 g per one kilogram of body weight. The experimental course lasted for 125 days. The other animal (No. 34) was given the dose of 100 mg per one kilogram of body weight at the beginning and the dose was increased gradually. The total dose given amounted to 4.9 g per one kilogram of body weight. The animal was killed after a course of 34 days.

The organs were fixed in 10 per cent formalin solution and the paraffin sections from the various kinds of organs were prepared by ordinal procedure.

The distribution of chinoform was examined in these tissue sections.

d) Application to the liver section in the human autopsy case of chinoform intoxication.

An adult patient (7), 40 years old, was admitted to Nakajima Hospital in Tokyo because of diarrhea and paraesthesia of both legs at the end of January, 1970. He was administered 2.0 g enterovioform per day for the treatment of diarrhea, but he became worse gradually and died on the 10th of July, 1970. A total dose of 89 g enterovioform was given. The technique of histochemical demonstration of chinoform was tried in the sections of liver tissue in this case.

B Methods: In test tubes, combinations of various kinds of metals with chinoform which were dissolved in Tween 80 solution were examined and copper, iron and silver were used as metals combined with chinoform in these metals (Table 1).

The metals which combined with chinoform and sedimented in tissues were stained histochemically to visualize successfully under the microscope (Table 2).

In the preliminary experiment, three procedures were determined successfully using copper, iron and silver.

a) A technique using copper-chinoform chelation mechanism.

1. Cut a fresh tissue by cryostat microtome, or prepare a paraffin section.
2. Immerse in 10 per cent Tween 80 solution containing 2 mM copper sulfate at 37°C for 2 hours.
3. Wash in running water for 30 minutes and then in distilled water.
4. Incubate overnight in the newly made mixture composed of 2 ml of 0.1 per cent rubeanic acid alcohol solution and 10 ml of 5 per cent sodium acetate solution at 37°C.
5. Wash in distilled water several times.
6. Stain nuclei with nuclear fast red.
7. Wash in water, dehydrate, clear and mount in balsam.

Control procedure: Okamoto and Utamura's rubeanic method (17) was performed as the control procedure. This method was used to stain copper which chelated with chinoform and sedimented in a tissue, so it had a possibility of demonstrating the presence of copper which existed naturally in tissue. Copper, which chelated with chinoform, had to be differentiated from that which existed naturally in a tissue.

b) A technique using iron-chinoform chelation mechanism.

1. Cut a fresh tissue by cryostat microtome, or prepare a paraffin section.
- 2.

TABLE 1. Chelating agents for chionoform dissolved in Tween 80

Chemicals	Solubility in Tween 80	Precipitation	Coloration of Reaction
MgCl ₂	good	-	non
ZnCl ₂	"	-	non
Co(NO ₃) ₂	"	-	non
FeCl ₃	"	- (+)	green
P ₂ O ₅ ·24H ₂ O	"	-	non
HgCl ₂	poor	++	yellow
SnCl ₂	good	-	non
AgNO ₃	"	+++	yellowish
BaCl ₂	"	-	non
CuSO ₄	"	++	yellow
NiCl ₂	"	-	non
K ₂ Cr ₂ O ₇	"	-	non

Iron and chionoform reacted well and rapidly but did not almost precipitate.

TABLE 2. Mechanism of histochemical method of chionoform

Immerse the section in the 10 per cent Tween 80 solution containing 0.3 mM iron chloride at 37°C for one hour. 3. Wash in distilled water several times. 4. Immerse in the newly made mixture containing equal volumes of one per cent potassium ferrocyanide solution and one per cent hydrochloride acid solution for 30 minutes. 5. Wash in distilled water. 6. Stain nuclei with nuclear fast red. 7. Wash in water, dehydrate, clear and mount in balsam.

Control procedure: Perls-Stiedas Berlin blue reaction was performed as a control procedure. Iron which chelated with chinoform and sedimented was stained by this method. Iron which chelated with chinoform and sedimented had to be differentiated from that which existed naturally in a tissue.

c) **A technique using silver-chinoform chelation mechanism.**

1. Cut a fresh tissue by cryostat microtome, or prepare a paraffin section. 2. Immerse the section in 10 per cent Tween 80 solution containing 3 mM silver nitrate at 37°C for two hours. 3. Wash in distilled water. 4. Incubate in the newly made mixture composed of 2 ml 0.1 per cent rubeanic acid alcohol solution, 2 ml of hundredfold diluted solution of Japanese pharmacopoeia ammonium solution and 100 ml of diluted water. 5. Wash in distilled water several times. 6. Stain nuclei with nuclear fast red. 7. Wash in water, dehydrate, clear and mount in balsam.

Control procedure: Okamoto, Utamura and Akagi's rubeanic acid method (18) was performed as control procedure. This method was used to stain silver which chelated with chinoform and sedimented. It was necessary to differentiate the silver from that which existed naturally in tissue.

RESULTS

1. *The histochemical demonstration of chinoform hydrolyzed from chinoform glucuronide by β -glucuronidase and precipitated in the tissue section*

Chinoform hydrolyzed from chinoform glucuronide as the substrate by β -glucuronidase activity deposited *in situ* in the liver cells. This substance was chelated with a metal and its presence was demonstrable by staining the metal using the indirect histochemical process.

Practically, this precipitated chinoform in the tissue cells was clearly demonstrated in the form of blackish green granules using the technique of the copper-chinoform chelation mechanism, sky-blue granules with iron-chinoform chelation mechanism, and blackish brown granules with silver-chinoform chelation mechanism respectively (Fig. 1).

FIG. 1. Chinoform is shown as granules at the sites of lysosomes in the cytoplasm of liver cells by the histochemical technique using iron-chinoform chelation in liver section in which chinoform was deposited enzyme-histochemically by β -glucuronidase.

FIG. 2. Autoradiography in liver section of a rabbit four hours following administration of ^{131}I -chinoform.

FIG. 3. Chinoform is shown as granules presumably at the sites of lysosomes, in liver cells and Kupffer cells, by the histochemical technique using copper-chinoform chelation in a serial section of Fig. 2.

FIG. 4. Chinoform is shown by the histochemical technique using silver-chinoform chelation in serial liver section of Fig. 2.

The enzyme-histochemically demonstrable β -glucuronidase reaction which used naphthol AS-BI- β -glucuronide as the substrate was seen to be similar to the deposits in granular form. The reaction products presumably appeared in the lysosomes in liver cells as well as Kupffer cells.

Chinoform-metals chelation complexes were not seen well under the microscope in the tissue, so metals chelated with chinoform and precipitated, were stained with each of the histochemical methods and chinoform was demonstrated to be present indirectly (Table 2).

When these techniques are used to histochemically demonstrate the presence of chinoform chelated with a metal, there may be a probability of metals distributed naturally in the tissues. Therefore, the metals which existed naturally in tissue cells must be differentiated from the metal bound with chinoform. For this purpose, it would be necessary to compare with the histochemical staining of copper, iron and silver in the control serial sections respectively. In many cases, they can be differentiated by applying the control procedure.

Copper, iron and silver staining methods were performed respectively as control procedure and these metals could not be found in these materials at all. So, what was demonstrated was thought positively to be chinoform.

However, it has been confirmed that many metals exists in not only pathological but physiological state in tissues, so sufficient control procedure must be performed.

2. *The comparison of the findings of autoradiography and the histochemical demonstration in tissue sections of rabbits administered ^{131}I -chinoform*

Following autoradiography of the tissue sections of animals treated with ^{131}I -chinoform the autoradiograph was compared with the serial sections stained by the devised techniques for histochemical demonstration of chinoform.

The changes of the labeling index in autoradiographs of the tissue sections are illustrated in Table 3. In the liver, autoradiographic silver grains were mainly seen on the cytoplasm of liver cells (Fig. 2). The grains were not seen in the Kupffer cells as much as in the liver cells in the early stage, but there appeared to be relatively more 2 hours after the treatment. In the kidney, the grains were found mainly in the epithelial cells of proximal convoluted tubules, where they appeared in large numbers at 1 hour following the treatment. The grains were seen to be few in the epithelial cells of the other convoluted tubules. In the spinal cord, the grains were localized in glial cells of both grey and white matters, but they could not be found in nerve cells. In the nerve fibers of the peripheral nerves, a few grains were found, but in the Schwann cells of the peripheral nerves, even fewer were found.

On the other hand, in the serial tissue sections of the rabbits administered with ^{131}I -chinoform, chinoform would be demonstrated to be present histochemically with three techniques using copper, iron and silver. In the liver, chinoform was demonstrated to be present in the form of granules in the cytoplasm which presumably were lysosomes (Figs. 3, 4). In the Kupffer cells, it was localized in the granular form to the similar region of cytoplasm as in the liver cells. In the kidney, it was also found in the similar form of granules in the cytoplasm of tubular epithelial cells. This localization had a tendency to be found near the brush border, but it was not related to the lapse of time. In the spinal cord, it was demonstrable

TABLE 3. *Histochemical and autoradiographical distribution of chionoform having radioactive iodine in rabbits*

	Autoradiographical distribution			Histochemical distribution
	1 hr.	2 hrs.	4 hrs.	
Liver :				
Liver cell	++	+++	+++	+++
Kupffer cell	(+)	(+)	±	+ - +++
Epithelial cell of bile duct	(+)	++	(+)	-
Kidney :				
Glomerulus	+	(+)	(+)	-
Proximal convolution	+++	+++	++	+++
Loop of Henle	++	++	++	+
Distal convolution	(+)	(+)	(+)	-
Spinal cord :				
Grey substance				
Nerve cell		±	-	-
Glial cell		+	+	(+)
White substance				
Glial cell		±	±	(+)
Nerve fiber		+	+	-
Canaliculus cent.		+	+	-
Peripheral nerve :				
peripheral nerve fiber		+	++	(+)

as the small granules in the glial cells. In the peripheral nerves, it was detected in the form of the same granules in their axons and in Schwann cells around the myelin sheath.

The distribution of ^{131}I -chionoform, which was shown by the autoradiography, coincided to a great extent with that of the chelated chionoform which was demonstrated to be present by the three techniques for histochemical demonstration of chionoform.

Furthermore, copper, iron and silver were not demonstrated to be present histochemically in the control procedure, so what was demonstrated to be present histochemically was considered to be chionoform.

By this investigation, it was confirmed that the devised techniques could demonstrate chionoform which was administered perorally.

3. *Histochemical demonstration of chionoform by applying the new techniques in the tissue sections of rabbits poisoned with chionoform*

The histochemical demonstration of chionoform was applied to the liver tissue of one chionoform-poisoned rabbit. The reactive granules demonstrated were detected in the cytoplasm of liver cells and Kupffer cells. They were localized in the granular form in the specific areas of the cytoplasm which presumably were lysosomal or phagosomal portions.

The histochemical reaction occurred in various organs except for the tongue, esophagus, stomach, pancreas, heart and adrenal glands. Chionoform was intensely demonstrable in the kidney, liver and cartilage. In kidneys, the reaction products were abundantly seen in the epithelial cells of the proximal convoluted tubules and weakly in the epithelial cells of distal convoluted tubules. There was no reaction in glomeruli.

The intensive reaction occurred in the cartilaginous cells and matrices of the bronchial and tracheal cartilages. This reaction was very striking in view of no reaction in the control sections. In the lungs, there were reaction deposits in the lining cells of alveolar walls. The splenic cell contained the reaction products in various intensities, but no reaction was observed in the other cell components.

In the digestive tracts, the epithelial cells contained the reaction deposits in the small and large intestines but not in the stomach and duodenum.

In the lymphatic tissue of the vermiform appendix, the reaction products were observed in some enlarged histiocytes and in the centers of lymphatic nodules. The muscle fibers of skeletal muscle contained the chionoform deposition granules. The nerve tissue also contained the granules of the reaction products in glial tissues as well as in the peripheral nerves, but no reaction occurred in nerve cells and ganglion cells.

In control procedure, copper and silver were not detected histochemically in any tissues. So, distribution of chionoform could be found by the two metal chelation methods in the tissues of the poisoned rabbits.

The distribution of chionoform was demonstrated by the ironchionoform chelation mechanism. However, abundant iron was also stained in both spleens and livers of rabbits of No. 22 and No. 34 by control procedure, so the distribution of chionoform was not clearly observed. What was stained by Berlin blue reaction was thought to be hemosiderin, therefore iron-chionoform chelation method and Berlin blue reaction control procedure were performed following treating sections by 5 per cent oxalic acid for 3 hours. However, clearer discrimination was not observed.

It was proved that iron chelation technique was not useful in tissues in which iron exists abundantly. In such tissues, chionoform could be demonstrated by using other metal chelation mechanism.

4. *The application of the techniques to the chionoform-poisoned human material*

Since the liver tissue from the body of a dead person, which was proved by chemical analysis to be poisoned with chionoform, was obtained, the histochemical reactions of chionoform were tried on this liver tissue. The demonstrable reaction products of chionoform, each showing histochemical coloration for metals, were deposited in similar granular form in the cytoplasm of liver cells as well as Kupffer cells, when the tissue sections were incubated in the reaction mixture of each method.

The iron chelation reaction appeared in similar regions of the cytoplasm in the liver and Kupffer cells, but the reaction products were more widely demonstrable in the cytoplasm of both kinds of cells than the other two techniques, while hemosiderin was detected by control procedure in the serial sections. Following pretreatment for three hours with 5 per cent oxalic acid solution, the specific reaction for chionoform occurred, while the control procedure did not reveal the iron reaction. This result was different from the aforesaid chionoform-poisoned rabbits.

The copper and silver methods were specific for the demonstration of chionoform as the control procedures did not reveal copper and silver.

In the case of this liver tissue, it was verified that copper and silver chelation methods histochemically demonstrated the presence of chionoform.

In iron chelation method, iron was found in various kinds of tissues by Berlin blue reaction as control procedure, and when pretreatment by 5 per cent oxalic acid was used, a relatively good result was obtained. However, the effort is now made to discover a more efficient iron chelation technique.

DISCUSSION

It has been clarified from the epidemiological, clinical and experimental results that SMON may result from poisoning by chionoform. However, no chionoform has been histochemically demonstrable in the poisoned tissues up to date, because there was no method for demonstrating it by use of the histochemical technique. Therefore, the histochemical technique for demonstrating chionoform had to be newly devised.

Chionoform has been known to be a derivative of 8-hydroxyquinoline (oxin) which easily chelates with metals. 8-hydroxyquinoline had been used in the histochemical fields, particularly in the case of demonstration of β -glucuronidase by Fishman and Baker (3), who, of course, improved this chelation method by the azo dye coupling technique. The derivative of 8-hydroxyquinoline as well as oxin has been known to chelate easily with metals (Hollingshead (5)). These chelating substances could be easily demonstrable in histochemistry by the use of such demonstrating metals.

However, various kinds of metals may exist in some tissues and the chemical substances which may chelate with metals could also exist in the tissues, as already discussed by many investigations (Tomsett (33), Okamoto *et al.* (17, 18), Wigglesworth (35), Timm (31, 32), Lillie (8), Lillie *et al.* (9), Barden (1)). On the other hand, the erratic factors in the metal precipitation had been investigated when some enzymes were histochemically demonstrated by the metal precipitation method (Takeuchi (27), Janigan and Pearse (6), Burstone (2)). It was clarified from the results of these investigations that some kinds of metals could be useful histochemically for demonstrating specific substances by the careful application of a certain metal and effective differentiation from erratic reactions (Takeuchi (29)).

Accordingly, the application of metals for histochemical demonstration of chionoform must be examined. Among the chelating metals, copper, iron and silver were picked up successfully for this demonstration. Among these metal chelating methods, chionoform-copper resulted in the best reaction which was

relatively specific for rubeanic acid. Iron and silver must be used by differentiating the erratic reaction.

It is known histochemically that silver is one of the metals to easily deposit in tissue. However, it was proved in this study that silver chelation technique was as specific as the copper chelation technique. On the other hand, in the cryostat tissue section of the rabbit in which chinoform was hydrolyzed from chinoform glucuronide and sedimented by β -glucuronidase, chinoform could be demonstrated to be present by the chinoform iron chelation method and iron was not detected by control procedure.

However, in tissue sections of chinoform poisoned man and rabbits, strong sediment of hemosiderin was seen, so it was difficult to differentiate iron, which chelated with chinoform and deposited, from iron of hemosiderin. Furthermore, Janigan *et al.* (6) mentioned that uncertain sediment occurred easily in Fishman and Baker's enzyme histochemical method of β -glucuronidase using the oxin-iron chelation mechanism and that the histochemical iron reaction was unstable. It was proved that in the iron chelation technique these tests should be conducted carefully and there was yet a problem in obtaining an accurate result.

On the other hand, chinoform has the property of being difficult to dissolve in water. Accordingly, a solution which chelated with metal was needed. It was found from this experiment that Tween 80 was very useful for making the chelation.

Chinoform has another property that the metal chelated chinoform produces fluorescence. Misumi and Sakurama (12) already found that chinoform chelated with gallium and aluminium would make the best fluorescence, and the histochemical fluorescence technique for demonstration of chinoform was devised. It was found that the localization of chinoform by the metal precipitation method coincided with the localization of fluorescence produced by the fluorescence technique. These facts probably prove that the devised techniques were very useful for histochemical demonstration of chinoform.

Practically, chinoform was demonstrated in various kinds of tissues of poisoned rabbits and in the liver of a human patient who was administered chinoform.

In the SMON, the pathological changes were mainly observed in the nervous system (Tsubaki *et al.* (34), Takasaki *et al.* (24), Hayase *et al.* (4), Matsuyama (11), Shiraki *et al.* (21), Oyake *et al.* (19), Tateishi *et al.* (30), Ohnishi (14, 15)), pancreas (Takeuchi *et al.* (28), Miyoshi *et al.* (13)) and muscle (Sakurama *et al.* (20)). This fact did not always coincide with autoradiographic distribution of ^{131}I -chinoform (Takahashi *et al.* (22, 23), Takasu *et al.* (25, 26)). It showed that there were specific relationships between toxin and different kinds of cells disturbed. It was thought that chinoform may specifically cause the pathological changes in peripheral nerve, pancreas and muscle even if the dose is smaller. Chinoform was not demonstrable or difficult to demonstrate in these tissues, while it was abundantly contained in liver and kidney which were not so greatly injured.

However, it was proved that the devised techniques utilized in this examination could detect chinoform histochemically. Moreover, chinoform could be demonstrated by these techniques in the liver, kidney, spleen, lung, appendix, large intestine and the others.

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