Sensitive detection of trace aluminium in biological tissues by confocal laser scanning microscopy after staining with lumogallion†

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This paper describes a method for the sensitive detection of aluminium in biological tissues by real time confocal laser scanning microscopy after staining with lumogallion. The method enabled detection of aluminium ≥9 μg g⁻¹ in bone and is more sensitive than the conventional histochemical methods with aluminon and solochrome azurine, etc. Lumogallion reacts specifically with aluminium to form a fluorescent complex so that the proposed method is useful for detection and identification of aluminium in tissues.

Keywords: Aluminium; lumogallion; confocal laser scanning microscopy; biological tissues

Our understanding of the relationship between the toxicity of aluminium and Alzheimer’s disease and the cause of aluminium-related bone disease attendant upon patients with chronic renal failure can be substantially enhanced if sites of sorption and localization of aluminium within the organism can be identified. The traditional methods of autoradiography and radioisotope identification are not possible since there are no suitable isotopes for aluminium. Highly sophisticated and sensitive methods of elemental microanalysis, e.g., electron energy loss analysis and energy dispersive X-ray microanalysis can be used for aluminium, although the sensitivity may not be adequate for some samples, and the effects of sample preparation (fixation, dehydration, embedding) on aluminium mobility need to be clarified. In some cases, simple histochemical stains can be used as an alternative. With regard to detection of aluminium in bone tissues, Ellis et al.1 reported that aluminium stain for bone tissues of mean aluminium concentrations of 7.5 μg g⁻¹ was negative and that bone tissue positive stain rate increased at aluminium concentrations of ≥22.0 μg g⁻¹ with aluminon and ≥19.50 μg g⁻¹ with solochrome azurine. Havas2 proposed a hematoxylin staining technique to locate sites of aluminium binding in aquatic plants and animals which had been exposed for 24 h to either 0.5 mg total aluminium per l or 1.0 mg total aluminium per l of synthetic water solution, but he did not give the detection limit. Morin reacts with aluminium to form a complex which fluoresces green, with an excitation wavelength of 420 nm, and an emission wavelength of 510 nm. However, information about aluminium staining with morin is scarce. Recently Tice et al.3 used fluorescence and confocal laser scanning microscopy after staining aluminium with morin to differentiate apoplastic and symplastic aluminium in root tips of aluminium-intoxicated wheat, but gave no data concerning the detection limit.

Lumogallion is known to react with aluminium in weakly acidic media to form a red complex which fluoresces a red colour (wavelength 580 nm) when exposed to 488 nm light. Based on this characteristic of the aluminium–lumogallion complex, fluorimetric methods for the determination of aluminium in various samples with lumogallion have been reported.4–7 In previous papers,5–7 we proposed the method of fluorimetric microscopic detection of aluminium in bone tissues after staining with lumogallion. In the present work, we observed aluminium at concentrations of ≥9.0 μg g⁻¹ in bone or liver tissue of rats, to which aluminium dosage was given by intraperitoneal (i.p.) injection, by a real time confocal argon laser (488 nm) scanning microscope after staining the aluminium with lumogallion. For comparison, aluminium concentrations in liver and bone tissue were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES).

Experimental

Instrumentation

For local detection of aluminium in biological tissues, a real time confocal laser scanning microscope (Meridian INSIGHT plus, Meridian, Okemos, MI, USA) was used. The excitation source was an argon laser (wavelength 488 nm). Fluorescent images were taken by a cooled charge coupled device camera and analyzed by an image analysis system (Meridian INSIGHT IQ system). Elemental analysis was performed with an ICP–atomic emission spectrometer (Thermo Jarrell Ash IRIS/AP, Franklin, MA, USA). A Shimadzu RF-5300PC spectrofluorimeter (Shimadzu, Tokyo, Japan) was used to check fluorimetrically the aluminium content of the lumogallion purified as described below.

Reagents

Lumogallion was purchased from Dojindo Laboratories (Kumamoto, Japan) and purified by recrystallizing two times from 50 °C ultrapure hydrochloric acid (Kanto Chemicals, Tokyo, Japan). The aluminium content of the purified lumogallion was below the detection limit (3.6 ng ml⁻¹) of the fluorimetric method proposed by Nishikawa et al.4 Aluminium stock solution for atomic absorption spectrometry (1000 mg l⁻¹) was obtained from Wako (Osaka, Japan) and an aliquot of this solution was used for preparation of working solutions. Acetate buffer (pH 4.0) was prepared by mixing 0.1 mol l⁻¹ acetic acid with 0.1 mol l⁻¹ sodium acetate. All chemicals used were of analytical-reagent grade unless otherwise stated.

Animals

The experiments were performed with 5 week old Wistar rats, comprising group I of rats with intact kidneys (n = 12) and group II of ⅔-nephrectomized rats with renal failure (n = 17). Group I consisted of three subgroups (A, B and C) and received a standard diet (A, control); 5% aluminium hydroxide in standard diet (B); or the same supplemented diet as in B plus 5% aluminium potassium sulfate, 1 mg per animal i.p. three times a week (C) for a period of 3 or 6 months. Group II rats with chronic renal failure which had been ⅔-nephrectomized one

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month previously were similarly divided into three subgroups (D, E and F) to receive the same regimen as in A (D), the same regimen as in B (E) or the same regimen as in C (F) for 3 or 6 months.

At the completion of the treatment period, the rats of both groups were bled under ether anaesthesia, followed by perfusion with a fixative solution and excision of organs and bones for assay and histological specimens.

**Lumogallion staining procedure**

Isolated specimens were fixed with 70% ethanol for 2 or 3 d at room temperature and immersed in pure ethanol for 2 or 3 d, followed by embedding in methyl methacrylate resin. One section of about 100 μm thickness was processed from each block with an Mc-411 microtome (Marto, Tokyo, Japan), and thin sections were produced manually.

Each group of thin sections were stained in a buffer solution (pH 4.0) containing 2.5 × 10⁻² mol l⁻¹ lumogallion for 1 h at 70 °C. The specimens were examined for aluminium using a real time confocal laser scanning microscope.

**Determination of aluminium in tissues by ICP-AES**

A portion (0.5–1.5 g) of bone or liver tissue was weighed into a silica beaker and decomposed with 10–20 ml of mixed acid (nitric acid : perchloric acid; 5 : 1) and evaporated to dryness. The residue was taken up with 0.2 ml of nitric acid and diluted with ultrapure water to 10 ml in a PTFE calibrated flask. Aluminium was then determined by ICP-AES. Instrumental conditions are summarized in Table 1.

**Results**

Table 2 presents the aluminium concentrations in tibiae and livers of rats with normal kidneys and those with renal failure. For the control group, the aluminium concentrations in the rats (A) with normal kidneys and the rats (D) with renal failure were 3.54 μg g⁻¹ and 4.80 μg g⁻¹ in tibia, and 0.82 μg g⁻¹ and 1.20 μg g⁻¹ in liver, respectively. On the other hand, deposition of large amounts of aluminium were found in the tibiae and livers of rats orally administered with aluminium (B, C, E and F).

In Figs. 1–4 the fluorescent images of the tibiae and livers of rats with normal kidneys and those with renal failure are shown. In the case of the control groups fluorescence was not observed for both the group with normal kidneys (A) and that with renal failure (D), while in the groups of rats fed a diet containing aluminium, intense fluorescence was observed for rats with normal kidneys and also those with renal failure, indicating abnormal aluminium deposition. Aluminium deposition was observed in tissues of the crural bones of aluminium-fed rats. Osteoid tissues were the sites where aluminium accumulation was frequently found. In the liver, aluminium was confirmed to be localized in the liver cells. The intensity of fluorescence observed correlated well with the aluminium content measured by ICP-AES.

The results described above indicate the usefulness of lumogallion as a staining agent for the detection of aluminium that occurs in trace quantities in biological tissues.

**Discussion**

**Evaluation of aluminium staining agents**

Aluminon, solochrome azurine, hematoxylin and morin have been used for the histochemical detection of aluminium in biological tissues. With regard to the detection limit of aluminium in bone tissue and the interpretation of results thereof, Ellis et al.¹ stated that tissue staining with aluminon or solochrome azurine was sufficiently, yet not entirely, specific for aluminium because iron, beryllium, etc., react with aluminon and solochrome azurine to form complexes also. In addition, they reported that aluminon stain for bone tissues of mean aluminium contents of 7.5 μg g⁻¹ was negative and that

![Figure 1](image-url)

**Table 1** Instrumental conditions for ICP-AES

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rf power supply</td>
<td>27.12 MHz</td>
</tr>
<tr>
<td>Operating power</td>
<td>1.15 kW</td>
</tr>
<tr>
<td>Nebulizer</td>
<td>Cross flow</td>
</tr>
<tr>
<td>Sample aspiration rate</td>
<td>0.5 ml min⁻¹</td>
</tr>
<tr>
<td>Argon flow-rate</td>
<td>15 l min⁻¹</td>
</tr>
<tr>
<td>Peak search window width</td>
<td>0.033 nm</td>
</tr>
<tr>
<td>Integration time</td>
<td>3 s</td>
</tr>
<tr>
<td>All emission line</td>
<td>396.15 nm</td>
</tr>
</tbody>
</table>

**Table 2** Aluminium concentrations (μg g⁻¹)* in livers and tibiae of rats with intact kidneys and 5/₆-nephrectomized rats

<table>
<thead>
<tr>
<th>Tissues/group</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.8</td>
<td>36.0</td>
<td>21.6</td>
<td>1.2</td>
<td>23.2</td>
<td>34.6</td>
</tr>
<tr>
<td>Tibia</td>
<td>3.5</td>
<td>10.9</td>
<td>9.1</td>
<td>4.8</td>
<td>21.8</td>
<td>10.1</td>
</tr>
</tbody>
</table>

* Average value for each group. + For A–F, see Experimental.
bone tissue-positive stain rate increased at aluminium contents of \( \geq 22.0 \, \mu g \, g^{-1} \) with aluminon and \( \geq 19.50 \, \mu g \, g^{-1} \) with solochrome azurine. However, the means and range of bone aluminium content are \( 7.60 \, \mu g \, g^{-1} \) and \( 1.5–13.0 \, \mu g \, g^{-1} \) for human control samples, respectively, and \( 15.1 \, \mu g \, g^{-1} \) and \( 1.5–113.4 \, \mu g \, g^{-1} \) for human renal failure samples, respectively. Thus interpretation based on stained color tone with aluminon or solochrome azurine seems to be difficult in the concurrent presence of iron.

Unfortunately information about the detection limits of aluminium in tissues using morin or hematoxylin staining is not available.

In the present experiment on rats using the lumogallion staining technique, the mean and range of tibial aluminium content were \( 2.56 \pm 1.38 \, \mu g \, g^{-1} \) and \( 1.58–3.54 \, \mu g \, g^{-1} \), respectively, for rats with intact kidneys, and \( 22.8 \pm 18.3 \, \mu g \, g^{-1} \) and \( 6.5–45.6 \, \mu g \, g^{-1} \), respectively, for aluminium-loaded rats with renal failure. Staining with lumogallion was invariably

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**Fig. 2** Cross section of liver from rats with normal kidneys stained with lumogallion and scanned with the laser confocal microscope, using 488 nm argon line for excitation. For A, B and C, see Experimental.

**Fig. 3** Cross section of the diaphyseal end of tibiae from \( 5/6 \)-nephrectomized rats stained with lumogallion and scanned with the laser confocal microscope, using 488 nm argon line for excitation. For D, E and F, see Experimental.

**Fig. 4** Cross section of liver from \( 5/6 \)-nephrectomized rats stained with lumogallion and scanned with the laser confocal microscope, using 488 nm argon line for excitation. For D, E and F, see Experimental.
positive for bones containing aluminium $\geq 9.0 \, \mu g \, g^{-1}$ and negative for those containing aluminium of $\leq 6.5 \, \mu g \, g^{-1}$. The resolution of the present method is about 0.2 $\mu m$ (almost at the limit of resolution of the light microscope). Since lumogallion reacts specifically with aluminium to form a fluorescent complex, it is a useful reagent for the detection and identification of aluminium in tissues.

Possibility of application to diagnosis of aluminium-related bone disease

The feature of the present method is that aluminium in control rat tibiae does not produce fluorescence, while aluminium deposited in bone by abnormal metabolism is effectively detected by fluorescence. Most of the aluminium-related bone diseases are expected to be concerned with the deposition of aluminium in bone. Therefore the lumogallion staining method may be applicable to the clinical diagnosis of aluminium-related bone disease.

References