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Research Article

LIVER DISEASE RISK OF XENOBIOTICS DUE TO PERCUTANEOUS ABSORPTION REVEALED BY NANO-PALDI IMAGING MASS SPECTROMETRY

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ABSTRACT

We analyzed localization of methyl 2-octynate (2-OAm) in sections of liver by nano particle assisted laser desorption/ionization (nano-PALDI) imaging mass spectrometry (IMS). 2-OAm was applied to mice skin and it permeated through the skin and accumulated in the liver. In livers of single dose mice, 2-OAm was delivered to the liver for 6 hours and excreted from the liver for 24 hours. On the other hand, in livers of long apply mice, 2-OAm was retained in the liver. Furthermore, we could be revealed that 2- OAm was accumulated in bile ducts by analyzing at a high resolution. In addition, CD8 staining indicated that an inflamed bile duct was observed. 2-OAm triggered the inflammation due to a coincident localization of 2-OAm and bile duct. This imaging approach is a promising technique for rapid quality evaluation of xenobiotics.

INTRODUCTION

There are many chemical materials called xenobiotics in our life; it is not classified for toxin or medicine. Most of them are passed strict safety test. However, it is unclear where xenobiotics are delivered[1]. Thus, we concern about the relationship between a diseases and our health after intake and application of skin of xenobiotics, regardless of our wishes. Methyl-2-octynoate (2-OAm) as a fragrance was wildly used for a cosmetics, hair dye and food flavorings (Figure 1)[2,3].

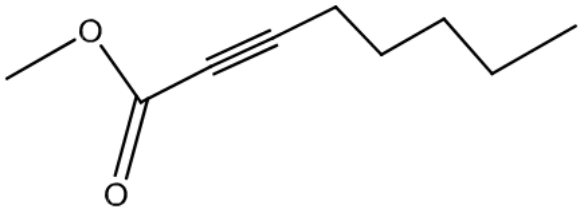

Figure 1 Chemical structure of methyl-2-octynoate

Recently, we have revealed that liver disease patients often were women and used hair dye[4]. Other report described that 2-OAm raised immune response against mitochondrial intima respiratory enzyme[5]. However, no evidence of route and destination of 2-OAm by skin application, visually. Generally, in such analysis like morphology diagnosis, Haematokylin and Eosin (HE) staining or immunostaining is common to evaluate various organs for complete examination. The analysis takes over 3days and gives us no direct information of existence and localization of target molecules such as medical drug and xenobiotics. Therefore, it is required developing of new technology and method to evaluate spatial information, visually. The way to determine the existence (what is it?), localization (where is it?) of biomolecules and their function in our human body has been a dream. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) is usually used to determine the molecular weights and structures of proteins, nucleic acids, and drugs. One of problems of MALDI is unsuitability of detection of low molecular weight target due to chemical matrix noises. We have developed a

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nanoparticle-assisted laser desorption/ionization (*nano*-PALDI) MS to avoid above problem[6,9].

Today, two-dimensional MS analysis of biomedical tissues by means of what is calledimaging mass spectrometry (IMS) has begun to be used to analyze analyte distribution[10]. Herein, we analyzed the disposition of 2-OAm visually by nano-PALDI imaging mass spectrometry (IMS)[6,7]. The purpose is to know whether 2-OAm is absorbed through percutaneous and where 2-OAm is carried in what form. When pharmacokinetics is analyzed, it is usual that target molecule was extracted from sample to analyze by chromatography and permeating image make by computer graphics. It can regard as indirect image. Whereas analysis of IMS, images is based on direct signal for sample sections, so it can regard as direct image. Images that can understand to see are easy to understand for everyone.

MATERIALS AND METHODS

Chemicals and materials

2-OAm, acetonitrile, methanol, $FeCl_2 \cdot 4H_2O$, $\rightarrow FeCl_2 \cdot 4H_2O$, carboxymethyl cellulose (CMC), haematoxylin and eosin were obtained from Wako (Japan). γ- aminopropyltriethoxysilane (γ-APTES) was purchased from Shin-Etsu Chemicals, (Japan). Female ICR mice (4 weeks old) were purchased from Crea (Japan). Care of and protocols for the experiments with animals were in accordance with the institutional guidelines of the National Institute of Health and the Animal Care and Use Committee (Fukui Prefectural University).

Animals

All experimentation with mice was conducted under protocols approved by Institutional Animal Care and Use Committees of the respective institutions.

Two different mouse models were prepared. For single application of 2-OAm mice(female ICR mouse, 20 weeks old), 2-OAm applied on mice back only once and sacrificed after 1, 3, 6, 24 hour, respectively. For long period of 2-OAm applied-mice (female ICR mouse, 25 weeks old), 20% 2-OAm applied on mice back once a week and it repeated for 40 weeks. Mice were sacrificed after 2hour passed for last application of 2-OAm. As control models, no application of 2-OAm mice were used. Mice were euthanized (each group contained 5–6 mice) and the liver was rapidly excised, rinsed in ice-cold saline, and weighed.

Preparation of Frozen sections of Liver

Each livers were embedded into 2% CMC after molding for cube and cut into serial sections (Single dose mice: 30 μm, Long applied mice: 25 μm) using blade in a cryostat (CM-3050 S; LEICA, Germany) at -20℃ and thaw-mounted on indium tin oxide (ITO) -coated slides for IMS. After thaw mounting, ITO slides were allowed to dry in a desiccator. The liver sections were coated with iron oxide-based nanoparticle (10 mg/ml) in methanol using an automatic spraying device (Image Prep, Bruker Daltonics). The liver were HE-stained. The section-mounted glass slide was immersed into a hematoxylin solution for 5 min and washed with tap water for 5 min. It was further immersed in 80% EtOH and then continuously immersed into an eosin solution for 1 min. After washing with tap water, the glass slide was continuously immersed into a series of EtOH solutions (70%, 80%, 90%, and 100%) for dehydration. The labeled glass slide was sealed with a coverslip using Entellan reagent.

Determination of 2-OAm profile in the liver by HPLC

The liver sample (0.6 g) of control or long application was added to 10 mL ofacetonitrile and suspended with homogenizer, respectively. The suspension was centrifuged (10 min, 10,000 rpm, 4 ºC) to remove insoluble materials. The volume of the supernatant was adjusted to 10 mL with acetonitrile. Extraction of 2-OAm from the liver was analyzed by high performance liquid chromatography (HPLC).
HPLC analysis-ODS column (Cosmocil, 5C18-AR-II, 4.6ID×250 mm) was used. Flow,

1.0 mL/min. isocratic elution, 0.1% TFA/water (A) and acetonitrile/0.1% TFA (B); 0-5 min, 40% B, 5-45 min, 40-80% B, 45-50 min, 100% B; for wash. The injection volume was 10 μl. target molecules were detected by 278 nm wavelengths.

Nano-PALDI-Based MSI

Nano-PALDI-based MSI was generated as described previously[6,7]. The iron-oxide based nanoparticle suspension was centrifuged, following which the supernatant fluid was sprayed as ionization assisting reagent on liver section using an air brush (nozzle caliber, 0.2 mm). MALDI mass spectra were acquired on an Ultrafle Xtrem MALDI- TOF/TOF (Bluker Daltniks). In order to detect the laser spot area, the sections were scanned and laser spot areas (1,000 shots) were detected with a spot-to-spot center distance (Single dose mice: 50 or 25μm) in each direction of the liver. The section surface was irradiated with 1,000 laser shots in the reflectron positive ion detection mode.

RESULTS AND DISCUSSION

Normal appearance of liver was explored until 10 weeks (wks) from the painting start. However, when it passed 15 wks, the damaged liver was observed (Figure 2).

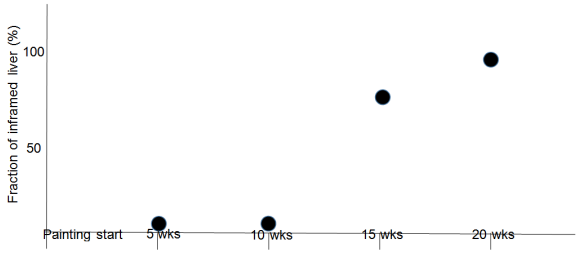

Figure 2 The relationship between application term and pathological appearance

Thus, we compared mouse liver that 2-OAm waspercutaneously applied for single and long term. MS measurement was achieved to detect 2-OAm from the liver. Nano-PALDI could preferentially ionize target molecule as sodium adducted form[6]. Sodium adducted signal of standard 2-OAm was mainly observed, indicating that we chose sodium adducted signal of 2-OAm on liver tissue section for MSI analysis. A number of high-intensity signals, including the signal at *m/z* 177.3 corresponding to sodium adducted 2-OAm, were detected in the mass spectrum obtained from the mouse liver seeped with nanoparticle (Figure 3).

From the liver of single application, the signal corresponding to 2-OAm was detected from 1, 3, 6, 24 hours. The brightness of imaging region increased with time.

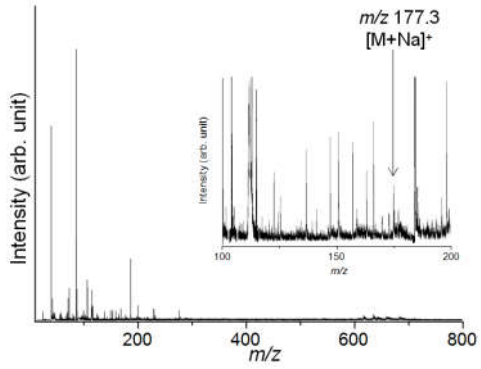

Figure 3 Mass spectrum of mouse liver.

Semi- quantitative analysis was performed by matching the MS intensities of liver. The ratio of signal intensity of the control and 1, 3, 6, 24 hours was 1:2:4:12:1. After 6 hours, the strongest signal intensity of 2-OAm was observed. After 24 hours, we marginally confirmed the signal of 2-OAm. From this result, we hypothesis that 2-OAm was derived to liver by percutaneous absorption and being carried via the bloodstream. After long time, 2-OAm passed out of liver and body by their metabolism (Figure 4).

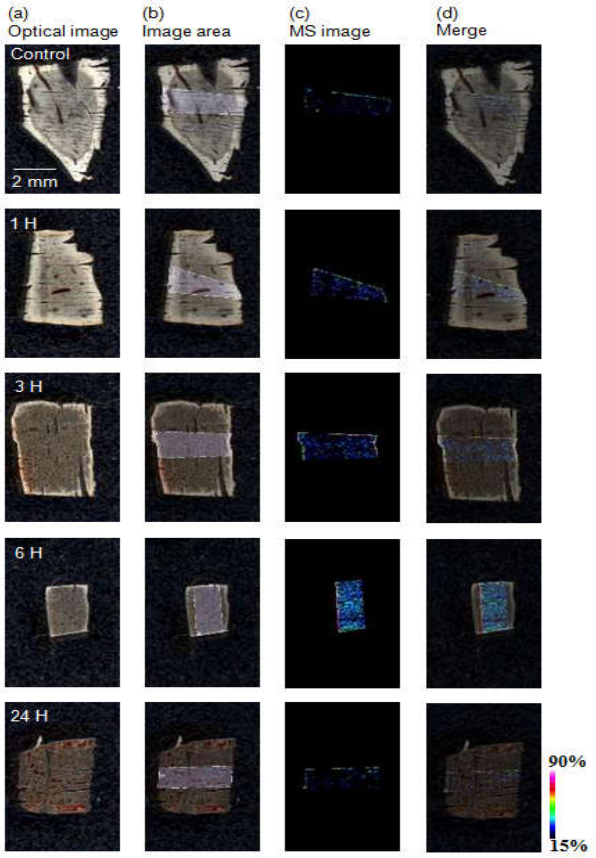

Figure 4 Nanoparticle-assisted laser desorption/ionization (Nano-PALDI) imaging mass spectrometry of 2-OAm from mouse liver for single application. Optical images of liver tissues (a) and imaging region (b). MS spectra reconstructed as ion images of 2-OAm (c) and merged image with optical and ion image (d).

Next, we investigated the effect of long period application of 2-OAm for liver. Figure 5 showed that 2-OAm was confirmed in liver although no signal was detected from control one. The ratio of signal intensity of the control and long apply mice was 1:3. Whereas 2-OAm was carried out of the body by single application, 2-OAm was retained in the liver by long period of application.

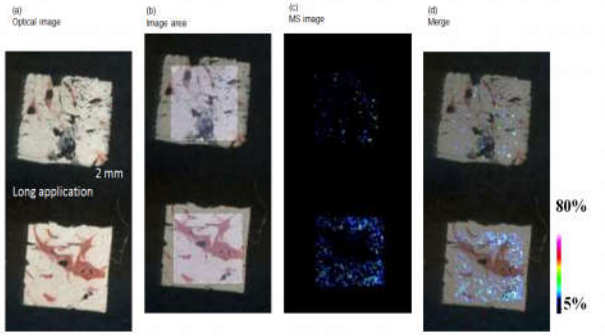

Figure 5 Nano-PALDI IMS of 2-OAm from mouse liver for long application. Optical images of liver tissues (a) and imaging region (b). MS spectra reconstructed as ion images of 2-OAm (c) and merged image with optical and ion image (d).

We investigated whether the increase in the amount of 2-OAm was caused by long application, quantitatively. Liquid chromatogram showed that the peak at 41.5 min. corresponded to 2-OAm. For the comparison of control and long application, total amount is 8.32 μg/g-liver for the sample of long application. For the control, we could not determine the amount due to no peak corresponded to 2-OAm. High spatial resolution of MSI was achieved to reveal the location which 2-OAm is accumulated. From HE stained image of control mouse liver, we could confirm the shape of bile duct (data not shown). On the other hand, 2-OAm-applied liver for long period did not show bile duct, clearly (Figure 6a). This area indicated CD8 positive (Figure 6b). CD8 was known to be important marker to analyze T-cell mediated inflammation. Infiltration of mononuclear cells were also observed in this area, therefore, we inferred that the shape of bile duct was destructed due to inflammation. IMS was achieved using serial section. The data showed that 2-OAm was accumulated at same location of inflamed region (Figure 6c and d). 2-OAm was also detected from yellow bile. This data proved that 2- OAm was accumulated at bile duct before its inflammation and predicable material of crash.

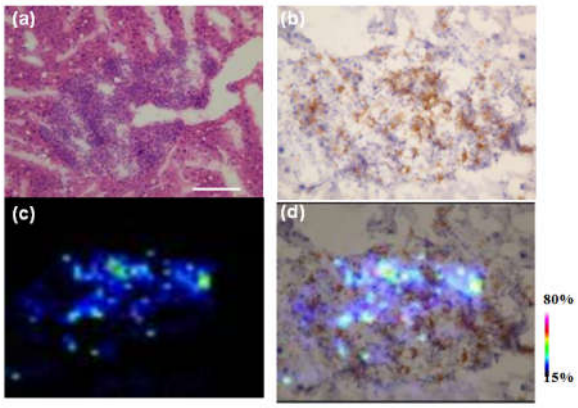

Figure 6 High-spatial resolution nano-PALDI IMS of 2-OAm from mouse liver for long period of application. HE (a) and CD8 (b) stained images of liver tissues of 2-OAm- appplied; MS spectra reconstructed as ion images of 2-OAm at 25 μm in mouse liver (c); merged CD8 and ion image(d). Scale bar is 200 μm.

These data indicated that excessive accumulation of 2-OAm which could be delivered to bile duct of liver by a percutaneous absorption and being carried via the bloodstream, and had relevance to occur the destruction of bile duct.

CONCLUSION

Nano-PALDI MS could detect 2-OAm as xenobiotics from 2-OAm-applied mouse liver. For Nano-PALDI IMS gives us the destination of 2-OAm, visually. In single application of 2-OAm to mice, 2-OAm was delivered to liver although 2-OAm was excreted out by metabolism system. In long period application of 2-OAm, 2-OAm could not be egested and retained in the liver especially in a damaged bile duct.

We hypothesize that the intradermally-administrated 2-OAm was derived to liver by vascular flow and occurred accumulation for bile duct, gradually. Accumulated 2-OAm was recognized as antigen. Thus a continuous 2-OAm applying made inflammation of bile duct by cell immunity. This research may suggested that the relationship between transdermal absorption of xenobiotics and a liver disease. Nano-PALDI IMS is useful technique for the identification and localization xenobiotics like chemical materials include cosmetics and medical drug.

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