Abstract:

Imaging mass spectrometry (IMS) requires the application of special sample pretreatment methods to enable the visualization of the target compounds with high sensitivity and high resolution. The ionization efficiency of matrix-assisted laser desorption/ionization is particularly low when used with IMS to detect steroid hormones. Additionally, steroid hormones cannot be analyzed with existing IMS pretreatment methods. This report describes an on-tissue derivatization method that enables the visualization of corticosterone and its successful IMS analysis with high sensitivity and high resolution using an iMScope TRIO microscope. Moreover, we describe the identification of the structural isomers of corticosterone by performing MS3 analysis with an ion trap.

Keywords: iMScope TRIO, imaging mass spectrometry, derivatization, corticosterone, structural isomer

1. Introduction

Imaging mass spectrometry (IMS) involves the direct mass analysis of a tissue surface to detect the imaging targets. IMS is known to be a molecular imaging method that shows the location, type, and amount of an imaging target without the use of target labeling. The existing methods used for sample pretreatment for IMS mainly involve the application of a matrix solution to the tissue surface, which forms a matrix-crystal layer that directly induces ionization. However, although this method is known to assist the visualization of phospholipids that exhibit polar regions and are present in large quantities on the tissue surface, IMS analysis often fails to visualize molecules that are not phospholipids. Consequently, some researchers consider that IMS technology is only capable of visualizing phospholipids. However, IMS can be used to detect the same target molecules as existing mass spectrometry methods with high sensitivity, provided that appropriate methods of sample pretreatment are employed. The techniques that enable this visualization are the two-step matrix application and on-tissue derivatization methods. We describe an IMS analysis approach that uses these two techniques to visualize corticosterone on rat adrenal tissue.

1-1. Two-Step Matrix Application

Very fine matrix crystals are known to increase the signal-to-noise ratio (SN) of the spectra obtained by matrix-assisted laser desorption/ionization (MALDI). The presence of very fine matrix crystals on the tissue surface is also expected to increase the SN in IMS and help improve the spatial resolution of the imaging results. However, the tissues analyzed by IMS are not normally washed before testing, and their surface includes large amounts of salts and contaminants. The application of a matrix to this type of surface causes the aggregation of the formed matrix crystals, which results in a very thin matrix layer in some areas. This type of inhomogeneity in the crystal layer affects image shading and makes data interpretation very difficult because what appears to be a change in the concentration of the target molecule could simply be caused by inhomogeneity in the crystal layer.

The two-step matrix application technique (hereafter termed the two-step method) was developed to improve this situation (Fig. 1). The first step of the two-step method involves the vapor deposition of a matrix crystal using an iMLayer system and the second step the spraying of the matrix solution. Vapor deposition with iMLayer results in the creation of very fine matrix crystals on the tissue surface. During the spraying of the matrix solution, these fine crystals on the tissue surface act as nuclei for the growth of matrix crystals.

First step: Vacuum vapor deposition of matrix using iMLayer

Second step: Spraying of the matrix solution using an airbrush

Fig. 1 Workflow of Two-Step Matrix Application Method
Images (Fig. 2) were captured with a scanning electron microscope to compare the morphology of the matrix crystals created by the two-step method and the conventional method of direct spraying (airbrushing) on the tissue surface. Although both images are displayed at the same magnification, the crystals created by the two-step method (Fig. 2a) are much finer and more densely spaced compared with those produced by the airbrushing method (Fig. 2b). The formation of this type of very fine and densely spaced crystal layer is known to increase the intensities of the spectral peaks of many target molecules, including compounds such as drugs and biological metabolites, by a factor of tens \[1, 2\].

This fine crystal layer is also required for performing high-resolution IMS analysis. High-resolution analysis (pixel sizes of 20 ≤ µm) with the very large matrix crystals that are formed on the tissue surface by the airbrushing method would result in imaging results influenced/alterd by the shape of these matrix crystals \[3\]. Based on the above, the two-step method is considered to be an essential pretreatment method for obtaining results with high sensitivity and high resolution.

### 1-2. On-Tissue Derivatization

On-tissue derivatization is a pretreatment method that further increases sensitivity and has attracted attention in recent years. The detection sensitivity of a liquid chromatograph mass spectrometer can be increased by performing derivatization in a solution \[4\]. Spraying the same derivatization reagent onto samples after the preparation of a tissue section also enhances the sensitivity of IMS. This treatment can even make previously undetectable molecules detectable. For this report, we chose Girard’s reagent T because it is an effective derivatization agent for the detection of steroids \[5\]. Corticosterone (\([M+H]^+\): 347.22) reacted quickly with Girard’s reagent T at room temperature and was then detected as derivatized corticosterone (\([M]^+\): 460.31) (Fig. 3). The derivatized corticosterone exhibited improved ionization efficiency owing to the inclusion of trimethylamine.

Derivatization reagent: Girard’s reagent T (Sigma-Aldrich) was prepared at a concentration of 10 mg/mL. Sample tissue: Sections of frozen rat adrenal gland were mounted on a glass coated with indium tin oxide (ITO) (Matsunami Glass: resistance 100 Ω, no magnesium aluminum silicate coating).

Matrix solution: Alpha-cyano-4-hydroxycinnamic acid (\(\alpha\)-CHCA, purity ≥98%, Sigma-Aldrich) was prepared at a concentration of 10 mg/mL. A mixture with a final concentration of 30% acetonitrile, 10% isopropanol, and 0.1% formic acid was used as the solvent.

Microscope image capture: Optical images of the samples were captured by an iMScope TRIO microscope before any sample pretreatment. Spraying of derivatization reagent: The derivatization reagent was sprayed onto the tissue surface using an airbrush (GSI Creos Procon BOY). The quantity that was sprayed was approximately 60 µL per tissue section. During the spraying process, we repeatedly dried the tissue surface after confirming that it was slightly wet. After all the derivatization reagent had been sprayed, the sample remained at room temperature for 90 min. Matrix application: After the completion of the derivatization reaction, vapor deposition of \(\alpha\)-CHCA was performed for 3 min at 250 °C, forming a thin layer of \(\alpha\)-CHCA on the tissue surface. The matrix solution was then sprayed onto the tissue surface with an airbrush. The quantity that was sprayed was 100 µL per tissue section, and the spraying was performed by the same method used for spraying the derivatization reagent. Separate airbrushes were used for the application of the derivatization reagent and the matrix. IMS analysis: We used an iMScope TRIO microscope. An IMS laser spot diameter of \(d = 2\) was selected for a pixel size of 25 µm, and \(d = 1\) for a pixel size of 10 µm. All IMS analyses were performed by MS/MS. The laser intensities and collision energies were optimized for each laser spot diameter to produce maximum peak intensities of the product ions. The optimized conditions were determined using spot analysis of a reference standard of corticosterone derivatized in solution.
3. Results

3-1. Comparison between Product Ion Spectra of Corticosterone Reference Standard and On-Tissue Analysis

The product ion spectra for the reference standard of corticosterone and the on-tissue analysis are shown in Fig. 4. Fig. 4a shows the product ion spectra of underivatized corticosterone. These spectra were obtained using the reference standard of corticosterone adjusted to a concentration of 10 mg/mL and placed on a glass coated with ITO. The mass spectra showed a protonated form at \( m/z \) 347.22, whereas the spectrum of the product ion at \( m/z \) 347.22 clearly exhibited a base peak at \( m/z \) 329.21. This product ion was attributed to corticosterone minus H₂O. A comparison with spectra obtained using the same analysis performed on adrenal tissue showed no peak at the \( m/z \) value of the peak. This result can be interpreted as evidence that the imaging of corticosterone is impossible without its derivatization.

Fig. 4b shows the results of the same analyses performed using derivatized corticosterone. The mass spectrum of derivatized corticosterone displayed a peak at \( m/z \) 460.31, which was understood to represent \([M]^+\). On selecting the peak at \( m/z \) 460.31 as the precursor ion and performing MS/MS, a base peak was detected at \( m/z \) 401.24. As shown in Fig. 4b, this peak was understood to be derived from the neutral loss of trimethylamine.

Subsequent analysis of the tissue produced a product ion spectrum with a high S/N that entirely coincided with the spectrum of the reference standard. These results demonstrate that on-tissue derivatization is an effective method for the detection of corticosterone. In addition to the peak at \( m/z \) 401.24 detected in the analysis of derivatized corticosterone, another main peak occurred at \( m/z \) 373.25, which represented corticosterone without -CO.

3-2. Imaging of Corticosterone in Adrenal Tissue

Using the experimental conditions described above, we performed on-tissue derivatization of the rat adrenal tissue and obtained data for this sample. The results of MS/MS imaging (\( m/z \) 460.31, then \( m/z \) 401.24) of the derivatized rat adrenal glands are shown in Fig. 5. Adrenal glands are known to consist of a layered structure that includes (from the inside out) the medulla, zona reticularis, zona fasciculata, zona glomerulosa, and capsule. Using the Imaging MS Solution analysis software designed for the iMScope TRIO, the results of MS/MS imaging were superimposed with optical images, showing that corticosterone accumulated in the zona fasciculata. The high-resolution analysis of a region encompassing the medulla, zona reticularis, and zona fasciculata determined a small amount of corticosterone in the medulla and accumulation of corticosterone in the zona fasciculata, which was the outermost layer of the analyzed region. Corticosterone is known to be produced in the zona fasciculata, and the implementation of IMS allowed the successful direct visualization of this production.
3-3. Applications of MS^n in Analysis of Biological Tissue

In addition to achieving high-resolution IMS using atmospheric-pressure MALDI for sample analysis under a microscope, the iMScope TRIO can also be used for MS^n analysis. Algestone (Fig. 6a) is a structural isomer of the steroid hormone corticosterone. The ability to distinguish structural isomers is important for accurate imaging of the distribution of corticosterone. Because the peak that we obtained by MS/MS using IMS with on-tissue derivatization corresponded to a molecule produced from the neutral loss of a trimethylamine, the existing IMS pretreatment methods did not distinguish between the different structural isomers of corticosterone. However, the iMScope TRIO can perform MS^3 analysis with an ion trap, which allows the indirect assessment of the existence of peaks produced by structural isomers present in the imaging results. This is performed by comparing the MS^3 spectra obtained from the analysis of a reference standard with those obtained from on-tissue analysis.

However, normal pretreatment does not produce peaks with sufficient intensity to perform on-tissue MS^3 analysis. In this experiment, we succeeded in performing on-tissue MS^3 analysis by combining the two-step matrix application and on-tissue derivatization methods to obtain peaks of sufficient intensity for MS^3 analysis. Fig. 7 shows the spectra obtained from the analysis of the base peak at m/z 401.24 that was determined by MS/MS. Although high-noise m/z regions are present in the spectra, the on-tissue MS^3 spectrum has a high S/N, and all the peaks obtained from the reference standard have sufficient intensity (Fig. 7, bottom). Based on these findings, the IMS results presented in Fig. 5 are considered to represent the distribution of corticosterone with very high accuracy.

4. Conclusion

This report described the two-step matrix application and on-tissue derivatization techniques that enable the visualization of target materials by IMS. Improvements in the sensitivity of the current IMS analysis can also be achieved by the development of sample pretreatment methods as well as via technological innovations in the applied instrumentation. We believe that as the scope of IMS applications increases, the demand for suitable sample pretreatment methods will also increase. We intend to develop a variety of such methods so that IMS can be used to its full potential.

References