Mass spectrometry Imaging (MSI) applications for ophthalmic drug discovery have recently gained growing interest especially for preclinical studies. Drugs for ocular treatment can be easily and quickly assessed for efficacy, safety, and toxicity using MSI. It permits to follow in the same experiment as the bio-distribution of your candidate and associated metabolites, as well as some potential modulation of efficacy or toxicity biomarkers (peptides, lipids, metabolites). Moreover, the high spatial resolution ability of the technique provides accurate localization of compounds in the anterior and posterior segments of the eye which are composed of several histological structures of just a few micrometers. Thus, MSI appears as a tool of choice for evaluating novel target or understanding mechanism of drug action on ocular diseases such as age-related macular degeneration (AMD), diabetic retinopathy, glaucoma, diabetic macular edema (DME), etc.

Experimental section:

- **Tissues:**
  New Zealand albino rabbits were treated with a 0.2% BAK solution (65.7% BAK C12 and 30.7% BAK C14) 2 drops per day. Rabbits were sacrificed, followed by eye enucleation at 1 month after the start of treatment. Eyes were stored at -80°C in tragacanth gum. Eye samples from human donors who suffered from glaucoma disease were obtained from the ‘Institut de la vision’ (Paris, France).

- **Sectioning:**
  Eye sections of 14 µm were obtained by cryostat CM3050S (Leica, Germany) or HM560 (Thermo Microm, Germany) and applied to indium tin oxide-coated conductive glass slides (Bruker Daltonik GmbH, Bremen, Germany). Silicon and glass slides were respectively used for ToF-SIMS analysis and Immunohistology study.

- **Matrix:**
  CHCA (10 mg/mL) in ACN/TFA 0.1%, (7:3, v/v) was deposited with an automatic spray system. After imaging, every section was washed with 100% methanol to eliminate the matrix before H&E (Haematoxylin/Eosin) staining. 1,5-diaminonaphtalene (DAN) powder (10 mg) was used and vaporized on tissue samples using a home built sublimation apparatus (150°C, 10 min, 0.6 mbar).

- **Mass spectrometry imaging:**
  Autoflex Speed LRF MALDI-TOF (Bruker Daltonik, Germany) with SmartBeam II laser. Positive mode (100-1000 Da) at 80 µm (for whole eye), 50 µm (for iridocorneal angle) spatial resolution. SRM mode was used on Autoflex to perform pseudo MS/MS measurement.
  Solarix 7T MALDI-FTICR (Bruker Daltonik, Germany) with smart beam II laser: positive mode (100-1200 Da) at 25 µm spatial resolution.

- **Software:**
  All presented MS images are from MultimagingTM software 1.1 (ImaBiotech, France).
In this application note we will present two examples. In the first example, MSI was used to characterize benzalkonium chlorides (BAK) spatial distribution and evaluate the physiopathological impact at the molecular level. BAK is the most commonly used preservative in eye drops, it is generally composed of benzododecinium (C12) and myristalkonium (C14). It is known to increase penetration of active compounds. However, numerous studies have reported its toxic effects on the ocular surface, especially in long-term treatments of diseases such as glaucoma [1,2].

Mass spectrometry imaging was also used to map BAK related ions in albino rabbit eye after installation of a mixed solution of BAK C12 (m/z 304.32) and BAK C14 (m/z 332.25). Thus, both ions were detected in different eye structures as shown on figure 1. BAK ions are mainly concentrated in the anterior part of the eye but also in small amount in the posterior segment. To provide more accurate information about BAK behavior in ocular tissues, we have focused our study on three important histological regions: the cornea (figure 2), the iridocorneal angle (figure 3) and near the optic nerve at the optic disc (figure 4).

Figure 1: (a) Optical image of H&E staining of rabbit eye 1 month after start of treatment and corresponding histological regions (left); (b & c) distribution of the two benzalkonium ions at lateral resolution of 80 μm.

The cornea is a clear, dome-shaped surface that covers the front of the eye. In our case, the BAK solution was instilled in the eye and consequently highly concentrated in this region. We can observe differences in penetration through cornea of two BAK ions in figure (2.b) depending on the structure of the compound. In fact the BAK C12 with the shorter carbon chain has a deeper penetration than BAK C14. It is well known in drug delivery area that the modification of the number of carbon in the fatty acid chain can modify the properties of the compound and thus improves or decreases its ability to cross tissue barrier. In this case, the MSI can provide supplemental information in terms of structural characterization of BAK ions. Moreover, optical and immunohistological images were used to provide complementary information about BAK impact on the cornea. H&E staining of figure (2.c) shows the degradation of CSE (corneal superficial epithelium) induced by BAK treatment. Epifluorescence results of figure (2.d) highlights apoptosis phenomena (Apoptotic cells in green) occurring within cornea/conjonctiva region due to BAKs action. All these results clearly indicate that BAK treatment induces damages to eye tissues at molecular and cellular levels.

Figure 2: Iridocorneal angle (a) MALDI-TOF Mass spectrum of standard collyrium solution (b) Fragmentation spectrum of BAK C12 ion, observation of two daughter ions at m/z 212 and m/z 91 (c) Zoom on iridocorneal angle area (d) MS image of BAK C12 ion distribution at lateral resolution of 50 μm (e) FAST-SRM MS image of BAK C12 fragment at m/z 212.
In glaucoma disease development, the **iridocorneal angle region** is highly important. In fact, the glaucoma is characterized by the increase of intraocular fluid pressure due to the obstruction of the trabeculum filter which is located within iridocorneal angle. Thus, the better understanding of the BAK implication in glaucoma treatment side effects is of high importance in this histological region. MSI enables the high resolution imaging of BAKs in iridocorneal substructures as shown in figure 3. MS and MS/MS (FAST-SRM mode) mass spectra obtained from this tissue region are presented in figure (3.a) & (3.b). We can observe on MS/MS spectrum the tropilium ion (m/z 91.05) and one reliable fragment of BAK C12 (m/z 212.24). This allows the univocal characterization of compounds followed on MS images. Moreover, it is possible to perform MS and MS/MS images (figure 3.d & 3.e) to provide a higher degree of confidence in the distribution of target molecule with the co-localization of parent and daughter ion. According to MS images from figure 3, we highlight here the accumulation of the BAK C12 ion at the sclerocorneal junction and near trabecular meshwork involved in aqueous humor outflow.

**Markers research for efficacy and safety investigations in human Glaucoma**

The second example deals with lipid composition of the different eye structures, especially of the posterior part of the eye, the **sclera/choroid/retina** regions. Specific lipids can be distributed differentially through retina layers and have consequently specific function in ocular biological organization. Several regions of the posterior part of the human eye could be implicated in the development of eyes diseases if they are impaired. The ability to identify molecular structures, such as lipid distribution within different parts of the eye, could be very useful in the research and identification of molecular biomarkers for eye diseases, like macular degeneration or retinitis pigmentosa.

High spatial resolution mass spectrometry imaging of the eye enables the visualization of histological regions within the different tissue layers. As an example, the posterior part of the eye’s mass spectrometric image at high spatial resolution (25 μm) is presented in figure 4. It shows the benefit given by a matrix sublimation method to yield high-quality molecular images with no analyte delocalization. Some contrast ionic species are used to differentiate histological structures of the eye. These ions are related to lipid species; but taking into account that there is no MS/MS data, the precise identification still remains difficult. Nevertheless, we are able to propose some potential lipids identification based on ImaBiotech’s proprietary databank for ion at:

- m/z 703.5640 (purple) as [M+K]+ which can be related to diglycerides; DG (39:1); Δm/z=0.7 ppm
- m/z 774.3514 (red) remains unknown
- m/z 834.5994 (yellow) as [M+H]+ which can be related to phosphatidylcholine; PC (40:6); Δm/z=0.6 ppm
- m/z 723.4912 (green) as [M+Na]+ which can be related to phosphatidic acid; PA (36:2); Δm/z=1.2 ppm
- m/z 881.6199 (blue) as [M+Na]+ which can be related to phosphatidylglycerol; PG (42:2) Δm/z=3.4 ppm

**Figure 4:** On the left side, the H&E stain of the posterior part of the eye shows numerous layers of the human eye: sclera, choroid, retinal pigment epithelium (RPE), photoreceptor (PR) layer, outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL) and vitreous humor (VH). On the black square, positive MALDI-FTICR images of the posterior part of the eye displaying partition of lipids: (a) DG (39:1); [M+Na]+; m/z 703.5640, (b) Marker 1; m/z 774.3514, (c) PC (40:6); [M+H]+; m/z 834.5994, (d) PA (36:2); [M+Na]+; m/z 723.4912, (e) PG (42:2); [M+Na]+; m/z 881.6199.
Moreover, we can perform an untargeted differential analysis between healthy and glaucoma human eyes in order to discover potential biomarkers related to the pathology. A statistical Welch t-test has been used to compare MS imaging data from 3 healthy and 3 pathological human eye sections using Multimaging™ software. The figure 5 shows the most significant molecular species identified by the statistical analysis on MS images.

A2E has been detected surrounding the posterior part of the eye alongside the retinal pigment epithelium (RPE).

It shows a significant up-regulation in glaucoma condition (Fold Change=4) which can be related to the fact A2E is known to increase the RPE cell death by apoptosis in eyes diseases [3]. PC(34:1) is also detected with a higher intensity in pathological condition (FC=12.5). Interestingly, PC(16:0/18:1) has been reported as up-regulated in human eyes donors with glaucoma [4]. Marker 2 is still unknown, but extremely down-regulated in eyes from glaucoma patient (FC=0.002).

Figure 5: Molecular images of biomarkers in healthy and glaucoma conditions performed on a MALDI-FTICR apparatus at 170 µm spatial resolution in positive ionization mode.

Finally, the biodistribution of drug can also be assessed in these different cellular layers of the retina and especially in the melanin rich region of choroid. MS imaging can provide valuable information on drug-melanin binding by evaluating the strength of the binding. In fact, this phenomenon can be controlled by adjusting MALDI matrix composition [5].

Conclusion

The combination of classical staining and immunohistology with cutting edge MSI offers new powerful tools to investigate the distribution of various compounds like amphiphilic eye drop excipients with known deleterious effects, and is therefore useful in pharmacological and toxicological preclinical studies. Thanks to high spatial resolution MSI ability, it is possible to follow a drug, drug metabolites, and potential biomarkers for determining efficacy and/or toxicity of treatments.

Thanks to MSI, ImaBiotech can provide reliable safety and toxicity information about ocular drug distribution or biomarker modulation in small histological regions of the eye.

Benefits

- High spatial (25 µm) and high spectral (R>500000) resolution imaging
- Molecular histology combines with classical staining or immunostaining techniques
- Preserving histological specificities of metabolite accumulation
- Biomarkers assessment within tissue

Keywords

- Ocular Drug Delivery
- Drug Formulation
- Mass Spectrometry Imaging
- Glaucoma
- Aged-related Macular Degeneration
- Biomarker discovery
- Toxicity
- Safety

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