Assessment of Drug Toxicity in Small Histological Structure of the Eye: Application of Mass Spectrometry Imaging in Ophthalmic Context.

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Introduction

Benzalkonium chloride (**BAK**), the most commonly used preservative in eye drops is generally composed of benzododecinium (C_{12}) and myristalkonium (C_{14}) (Figure 2) and 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**. Mass spectrometry Imaging (MSI) applications to ophthalmic drug discovery have recently gained growing interest especially for preclinical studies in **pharmacology** or **toxicology**. In this experiment, MSI is used to characterize the BAK spatial distribution and evaluate its **physiopathological impact** at the molecular level.

Experimental

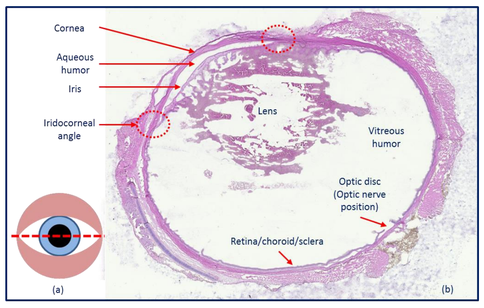


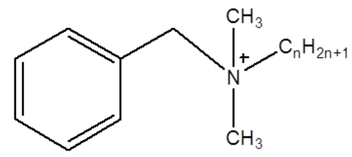
Figure 1: *(a) Cutting plane (b) Sagittal sections of rabbit eye with H.E. coloration and corresponding histological regions*

✓ **Animal preparation and sacrifice**

New Zealand albino rabbits were treated with a 0.2% BAK solution (65.7% BAK C_{12} and 30.7% BAK C_{14}) 2 drops per day. Rabbits were sacrificed, followed by eye enucleation at 1 month after the start of treatment. The eyes were stored at -80°C in tragacanth gum.

✓ **Tissue Sectioning**

Eye sections of 14 µm were obtained by cryostat CM3050S (Leica, 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.



n=12 BAK-C_{12}, m/z 304.30

n=14 BAK-C_{14}, m/z 332.33

Figure 2: *Molecular structure of benzalkonium ion.*

✓ **Matrix deposition**

The MALDI matrix, α-cyano-4-hydroxycinnamic acid (CHCA) was deposited with an automatic spray system. The matrix solution was CHCA (10 mg/mL) in ACN/TFA 0,1%, (7:3, v/v). After imaging, every section was washed with 100% methanol to eliminate the matrix before H.E. (Hematoxylin/Eosin) staining.

✓ **Instrument**

After matrix deposition, analyses of eye sections were performed in an AutoFlex Speed (Bruker Daltonik GmbH, Bremen, Germany). The AutoFlex Speed contains a YAG laser with a repetition rate of 1000 Hz. ToF-SIMS images were obtained using ToF-SIMS IV (IonToF GmbH, Münster, Germany). Structural analyse were performed using a MALDI LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany).

1. Iridocorneal angle: *Anterior part of the eye*

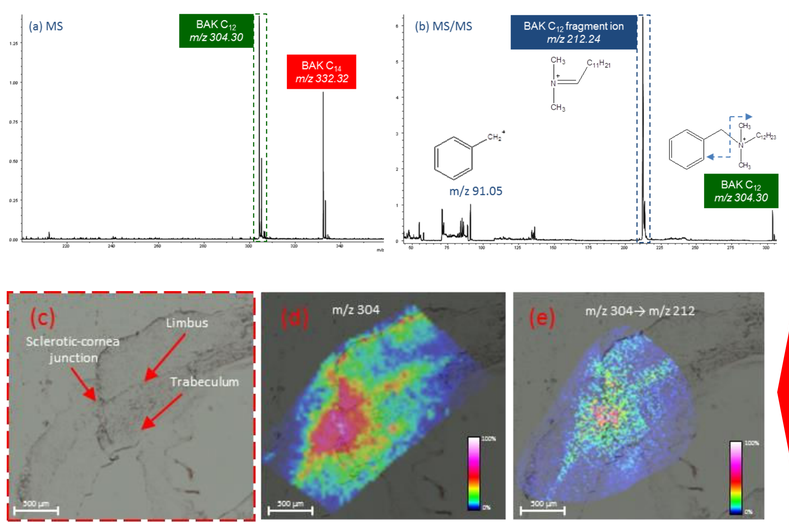
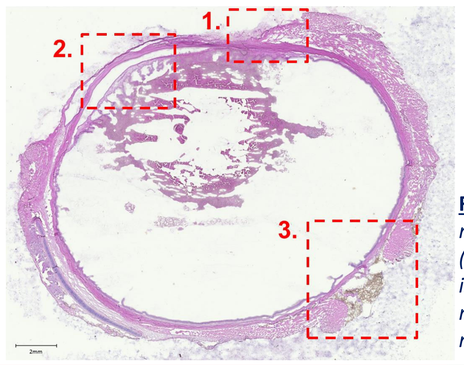


Figure 4: *(a) MALDI-ToF Mass spectrum of standard collyrium solution (b) Fragmentation spectrum of BAK C_{12} ion, observation of two daughter ions at m/z 212 and m/z 91 (c) Zoom on iridocorneal angle area (d) MS image of BAK C_{12} ion distribution at lateral resolution of 50 µm (e) FAST-SRM MS image of BAK C_{12} fragment at m/z 212*

- **Co-localization of parent and daughter ion.**
- **Univocal characterization of BAK distribution thanks to FAST-SRM (Single Reaction Monitoring) mode.**
- **Accumulation of the BAK C_{12} ion at the sclerocorneal junction and near trabecular meshwork involved in aqueous humor outflow.**

BAKs distribution in different ocular surface structures



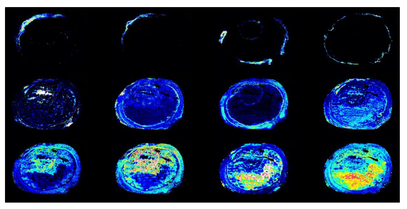
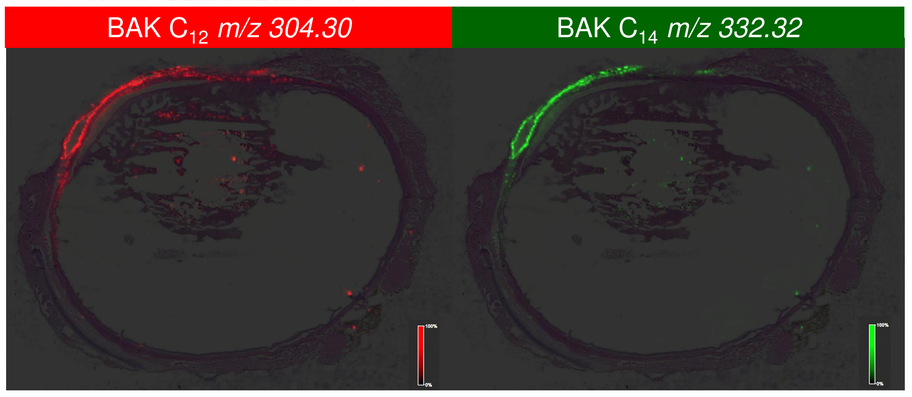


Figure 3: *Optical image of H.E. coloration of rabbit eye 1 month after start of treatment (left); distribution of the two benzalkonium ions (Red & Green images) and endogenous metabolites (polychromatic images) at lateral resolution of 80 µm.*



- **BAK ions were detected in different structures: (1) iridocorneal angle (2) cornea, iris, conjunctiva, limbus, retina and (3) near the optic nerve at the optic disc.**

2. Cornea: *Anterior part of the eye*

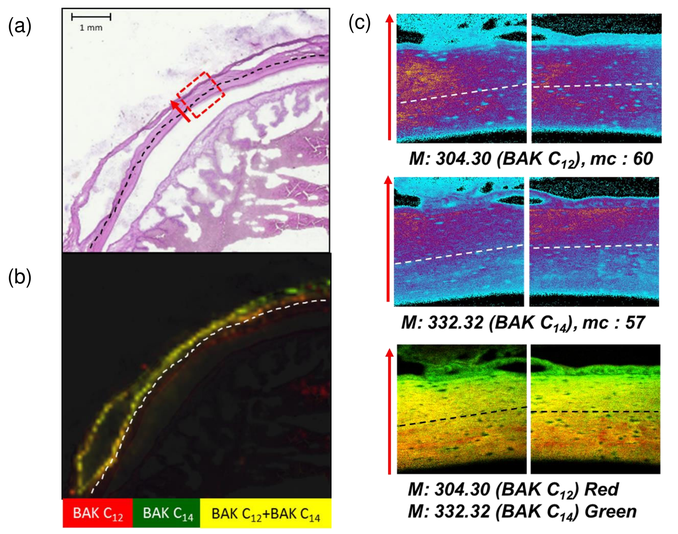


Figure 5: *(a) Zoom on cornea region (b) MALDI-ToFMS images of BAK C_{12} (red) and BAK C_{14} (green) ion distribution (superposition in yellow) at lateral resolution of 80 µm (c) ToF-SIMS images of the same ions and their superposition at a lateral resolution of 2 µm.*

- **Differences in penetration through cornea of two BAK ions following their structure.**
- **Complementarity of two mass spectrometric imaging techniques.**

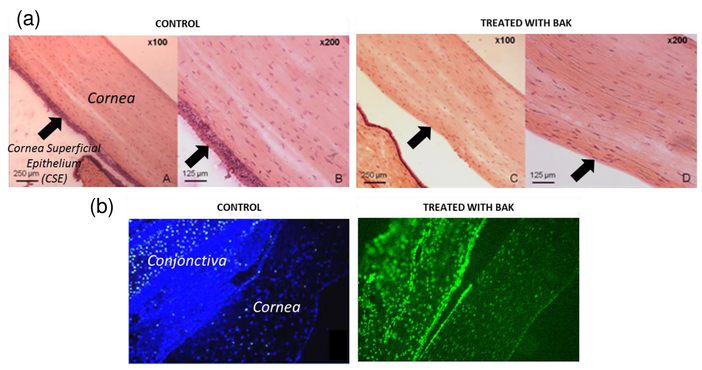
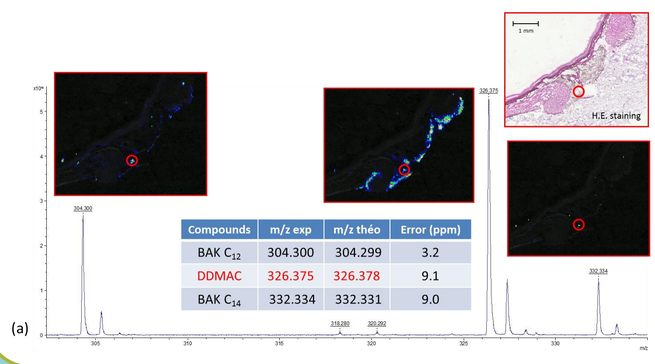


Figure 6: *(a) H&E Staining of control and treated rabbit eye section showing the degradation of CSE induced by BAK treatment and (b) Immunohistological images (Epifluorescence) highlighting apoptosis phenomena (Apoptotic cells in green) within cornea/conjonctiva region due to BAKs action.*

- **BAK treatment induces damages to eye tissues at molecular and cellular levels.**

3. Optic nerve region: *Posterior part of the eye*



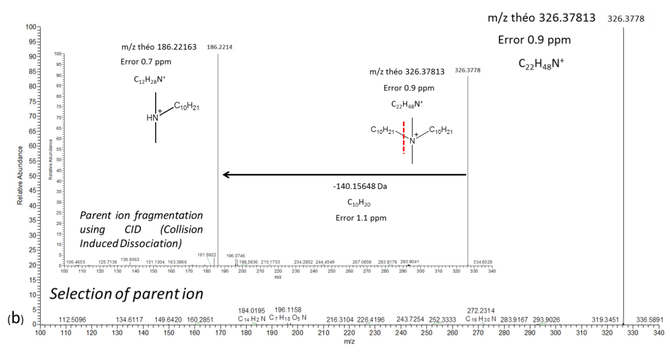


Figure 7: *(a) Mass spectrum related to red circle position on optic nerve region of MS image. (b) Selection and fragmentation of m/z 326 ion corresponding to didecyldimethylammonium salt using MALDI-Orbitrap MS/MS.*

- **Detection of BAK ions near optic nerve region.**
- **Characterization of unknown ion by MS/MS experiment and high precision mass measurement.**

Conclusions

✓ We report here the uses of mass spectrometry imaging in the study of the specific **benzalkonium chloride distribution** in the eye.

✓ These studies demonstrate that BAK could affect not only **ocular surface structures** (anterior part of the eye) but also the sensitive areas involved in the **glaucoma pathophysiology**, trabecular meshwork and in the **eye posterior segment** (retina and optic nerve region).

✓ **Classical staining** and **immunohistology** also permits to prove that BAK was associated with an infiltration of inflammatory (CD45 positive cells).

✓ The combination of these techniques with cutting edge MSI offers new powerful tools to investigate the distribution of various compounds like this amphiphilic eye drop excipient with known deleterious effects, and is therefore useful in pharmacological and **toxicological preclinical** studies.

Literature

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Acknowledgements

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