Distribution of chloroquine in ocular tissue of pigmented rat using matrix-assisted laser desorption/ionization imaging quadrupole time-of-flight tandem mass spectrometry

Yasuhiro Yamada¹, Kaji Hidefumi¹, Henry Shion²*, Motoji Oshikata³ and Yukari Haramaki³

¹DMPK Department I, DMPK Research Laboratory, Research Division, Mitsubishi Tanabe Pharma Corporation, Saitama, Japan
²Waters Corporation, Milford, MA, USA
³Nihon Waters, Tokyo, Japan

In pharmacology and toxicology, localization of the distribution of a drug molecule in its target tissue provides very important in vivo biological information. Traditionally, this has been examined using autoradiography (ARG). However, there are significant limitations in this application. One is the synthesis and use of radiolabeled compounds, the other is that the image generated expresses an undifferentiated mixture of the parent drug and/or its metabolites. The objective of the study was to define the specific distribution of the parent drug in rat ocular tissue containing melanin (e.g. the retina) using non-labeled chloroquine by MALDI Imaging tandem mass spectrometry (MS/MS). After single oral administration (at 20 mg/kg) of chloroquine, sections (10 µm) of rat eye tissue were prepared at 24 h. The MS system used was a quadrupole time-of-flight (Q-TOF) tandem mass spectrometer (MALDI Synapt™, Waters, Milford, MA, USA). Tissue sections were sprayed with CHCA (α-cyano-4-hydroxycinnamic acid, 5 mg/mL) in 80% acetonitrile (ACN) containing 5% formic acid (FA) using either a manual sprayer (airbrush) or an automated sprayer (TM-Sprayer™, HTX Technologies, Carrboro, NC, USA). Chloroquine was readily detected in the MS/MS mode by monitoring one of its major fragment ions (m/z 247.10) and imaged through the rat eye tissue. The image of the specific distribution within the retina in the rat eye tissue was confirmed, and found to be similar to autoradiograms after oral administration of ¹⁴C-chloroquine reported previously. Copyright © 2011 John Wiley & Sons, Ltd.

In drug discovery and development, it is critical to determine the metabolic fate of drugs absorbed in the body. This information (i.e. distribution and concentration of the drugs within the tissue and its rate of elimination) is important in assessing the efficacy and toxicity of a drug and can be obtained using a variety of techniques. Autoradiography (ARG) is a popular technique used by pharmaceutical companies¹⁻³ to display the tissue distribution of radiolabeled drugs (e.g. ¹⁴C- or ³H-) as a qualitative image, while liquid chromatography/tandem mass spectrometry (LC/MS/MS)⁴ is often used to determine the concentration of drugs in tissue samples. However, each technology has its limitations and although ARG offers visual spatial images of drugs to all tissues including microtissues such as the pituitary gland and the pineal gland, these images are associated with radioactivity (derived from a mixture of parent drug and/or its metabolites) and are not representative of the disposition of compounds associated with efficacy and toxicity. On the other hand, LC/MS/MS allows separate quantification of the parent drug and its metabolites without the use of radiolabels, but it is often difficult to determine the concentration of drugs in microtissues and its distribution within organ tissues such as the brain and kidney (cortex and medulla). Recently, matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-Imaging MS) has emerged as a new technique for drug discovery and development by several pharmaceutical companies⁵⁻¹². MALDI-Imaging MS combines the benefits of both ARG and LC/MS/MS, and is capable of mapping the non-radiolabeled distribution of both the parent drug and its metabolites using visual-spatial images. This has allowed the relationship between efficacy or toxicity with drug concentration to be evaluated in greater detail and at lower cost than ARG and LC/MS/MS combined, thereby providing a powerful addition to the toolbox for the spatial analysis of tissue distribution in drug discovery.

The biodistribution and accumulation in high level to specific tissues of the dosed drug and/or its metabolites can often lead to toxic events. For example, the basic drugs such as chloroquine,¹³ chlorpromazine¹⁴ and several beta-blockers¹⁵,¹⁶ have been reported to show major differences between drug transfers to the melanin-containing and non-melanin-containing tissues, due to high affinity of the drugs to melanin. It is well known that the elimination of drugs that bind to melanin is slower than that to other tissues. Also, chloroquine, an antimalarial agent, is associated with corneal deposits and pigmented retinopathy, which can lead to decreases in visual activity, a reduced visual field, and color-vision defects.¹⁷

* Correspondence to: H. Shion, Waters Corporation, Milford, MA, USA. E-mail: henry_shion@waters.com
Therefore, it is important to evaluate the specific distribution, residual life and accumulation for the candidate drugs in the melanin-containing tissue, to select drug candidates that are easily eliminated relatively early in drug discovery. In this study, MALDI-Imaging MS is applied to examine the possibility to determine the specific distribution of chloroquine to the melanin-containing tissue (in the retina) in the ocular tissue.

In order to obtain reliable and reproducible data for the selection of drug candidates early in drug discovery by MALDI-Imaging MS, it is important to deposit matrix solution uniformly on the tissue surface. The simplest approach is to use a pipette (droplet deposition) to manually deposit the matrix solution to the region of interest on the organ tissue surface. However, spatial information and reproducibility is poor and a more homogenous layer of crystals is often required. Typically, inexpensive hand-held aerosol sprayers or air brushes are used, but these also suffer similarly limitations due to difficulties generating homogenous droplet sizes and person-to-person variability. An alternative to overcome the above limitations is to automate the matrix deposition process. Several commercial platforms have been developed and include the chemical inkjet printer or ChIP (Shimadzu Biotech), ImagePrep (Bruker Daltonics Inc.) and, as used in these experiments, the TM-Sprayer (HTX Technologies Inc., Carrboro, NC, USA).

The TM-Sprayer is an easy-to-use, versatile spraying system that provides an automated process for the preparation of MALDI plates. The automated sprayer produces a very uniform and consistent coating crucial for reliable imaging. The deposition image obtained from the TM-Sprayer is compared with those obtained from the manual airbrush in order to examine the utility of the automated sprayer.

In this work, the proposed MALDI-Imaging MS method and automated matrix application techniques produced highly reproducible imaging results that revealed the localization of chloroquine in rat ocular tissue to the melanin-containing tissue of the uveal tract.

**EXPERIMENTAL**

**Materials**

α-Cyano-4-hydroxycinnamic acid (CHCA) and chloroquine diphosphate were purchased from Sigma (St. Louis, MO, USA). Formic acid (FA) and trifluoroacetic acid (TFA) were from Fluka (Taufkirchen, Germany). Acetonitrile (optima grade) and methanol (optima grade) were both from Fisher Scientific (Fair Lawn, NJ, USA). Water used was purified water from a Mini-Q system (Millipore, Bedford, MA, USA). All other reagents and solvents used were of the reagent grade commercially available.

**Animals**

The animals used in this study were male Brown Norway rats (Charles River Japan, Inc., Shiga, Japan) aged 6 weeks. The rats were not fasted before oral administration and drinking water was supplied ad libitum. All procedures for the animal experiments were approved by the Animal Ethics Committee of Mitsubishi Tanabe Pharma Corporation.

**Dose preparation and administration**

Chloroquine diphosphate was dissolved in purified water to prepare solutions of 20 and 100 mg/5 mL. This solution was administered by gavage at a dose of 5 mL/kg with a stainless steel stomach tube. Also, only purified water was administered to rat as vehicle.

**Tissue preparation**

Twenty-four hours after oral administration, the rats were subjected to laparotomy under anesthesia and were euthanized by exsanguinations from abdominal aorta with a syringe. Ocular tissues including hardier gland were immediately removed from administered rat of chloroquine or vehicle. The isolated ocular tissues were embedded in the Tissue-Tek O.C.T. compounds (Sakura Finetek Japan, Tokyo, Japan) and then were frozen and stored at −80°C until preparation of cryosections. The tissues were sliced using a Cryostat (Leica CM-3050, Leica Microsystems Inc., Bannockburn, IL, USA) at a tissue thickness of 10 μm at −18°C. The slices were mounted onto microscope glass plates (non-coating) using electrostatic force and then were dried by ventilation at the room temperature. To confirm the structures of eyes such as retina, iris, pupil and cornea, one of the plates was applied to hematoxylin-eosin stain.

**Matrix solution**

Four varieties of matrix solution was prepared: (1) CHCA was dissolved in 50% acetonitrile aqueous solution (v/v) including 0.05% TFA to prepare a solution of 10 mg/mL (Matrix-A), (2) CHCA was dissolved in 80% acetonitrile aqueous solution (v/v) including 1% TFA to prepare a solution of 10 mg/mL (Matrix-B), (3) CHCA was dissolved in 80% acetonitrile aqueous solution (v/v) including 5% FA to prepare a solution of 10 mg/mL (Matrix-C), and (4) CHCA was dissolved in 80% acetonitrile aqueous solution (v/v) including 5% FA to prepare a solution of 5 mg/mL (Matrix-D).

**Matrix application**

Two methods were used to apply matrix solution to the tissue sample surface: manual airbrush and automatic sprayer. In the manual method, airbrush, (Iwata studio series compressor fitted with an Iwata Eclipse gravity feed airbrush; Portland, OR, USA) was used to deposit the matrix solution on the tissue surface. In this method, the tissue was held vertically on a panel arranged in a fume hood. With the compressor pressure set at 40 psi, the matrix solution was continuously applied to the tissue horizontally by passing the airbrush from left to right and top to bottom (in alternation) over the tissue. While spraying the airbrush was held at approximately 40 cm away from the tissue. The surface of the tissue section was then allowed to dry fully in ambient air (1 min) to avoid sample wetting. This was repeated until 15 mL of matrix had been applied. With the automatic sprayer, the TM-Sprayer from HTX Technologies (Carrboro, NC, USA) was used to deposit the matrix solution on tissue surface. In the TM-Sprayer, a constant flow of heated sheath gas (N2, set at 10 psi) is delivered conjointly with the matrix solution spray. The temperature of the sheath gas was kept at 110°C. The solvent pump system used was a Smartline P1000 (Knauer,
Berlin, Germany) operated at a flow rate of 0.25 mL/min. The TM-Sprayer software was used for the system operations.

**Mass spectrometry**

Digital scans of the tissue section were obtained prior to the imaging experiment using HP ScanJet G4010 (Hewlett-Packard Co., Palo Alto, CA, USA) and imported into MALDI imaging Pattern Creator software (Waters Corporation, Manchester, UK) where the area to be imaged was selected. MALDI-MS analysis was conducted using a quadruple time-of-flight (Q-TOF) mass spectrometer equipped with a MALDI source (MALDI SYNAPT HDMS, Waters Corporation, Manchester, UK) operating in positive ion V-mode. The laser used was a frequency-tripled Nd:YAG laser (355 nm), firing at 200 Hz at the energy level of 250 (a laser energy level of 500 corresponding to about 100 μJ). MALDI MS data were acquired by moving the tissue sections in a raster pattern on an x/y stage relative to the laser position, which remained fixed. Spatial resolution was of 150 μm, and a total of 400 laser shots (2 s) were acquired per pixel. Argon was used as the collision gas. The trap collision cell voltage was set to 6 eV in MS mode and 22 eV in MS/MS mode, respectively, while the transfer voltage was kept at 4 V. The precursor ion was set at m/z of 320.18 for chloroquine for all MS/MS experiments. After the data acquisition, the resultant MassLynx data file (raw file) was converted into an .img file using MALDI Imaging converter software (Waters Corp., Manchester, UK) and visualized with BioMap 3.7.5.5 (Novartis, Basel, Switzerland).

**RESULTS AND DISCUSSION**

**Optimization of MS/MS instrument parameters**

The MS/MS instrument parameters were optimized prior to the measurement of chloroquine-administered tissues. Chloroquine aqueous solution (1 μL) at a concentration of 5 μg/mL was deposited on blank tissue surface which had been administrated with a vehicle, the tissue was dried in ambient temperature before 10 mg/mL CHCA solution as matrix was applied on top. The tissue sample was then loaded into the MALDI instrument to optimize the instrumental parameter settings. The chloroquine [M+H]+ ion is observed at m/z 320.19. The MS/MS spectrum of chloroquine contains a characteristic fragment ion at m/z 247.10 corresponding to the loss of the dimethylamine group from the parent molecule (Fig. 1). Five different collision cell energy settings (CID = 15, 18, 22, 25 and 28 eV) were tested and m/z 247.10 was found to optimize at 22 eV.

2,5-Dihydroxybenzoic acid (DHB) was also tested as the matrix for this study. However, under the same sample preparation and experimental conditions, CHCA produced much higher signal intensities for both the precursor ion at m/z 320.19 and fragment ion at m/z 247.10. Therefore, CHCA was chosen as the matrix for this study. The terms Matrix-A, -B, -C and -D are used to describe four matrix solutions with different solvent composition and buffer addition, with which CHCA was used as the matrix for all.

**Histological confirmation of ocular tissue**

Histological confirmation of ocular tissue cryosections in pigmented rat was carried out prior to MALDI-Imaging MS. The microphotograph obtained via the hematoxylin-eosin staining of the cryosection of the ocular tissue including the harderian gland is shown in Fig. 2. Microscopically, the functional tissue of eyes (i.e. retina, iris, pupil and cornea) was confirmed in the cryosection. Tissues such as the retina and the choroid are melanin-rich.

**Proof of the specific distribution of chloroquine to melanin-containing tissue by MALDI-Q-TOF**

Optimization of matrix solution

Applying Matrix-A using the manual airbrush. Chloroquine was orally administered to rats at doses of 20 mg/kg (conventional dose level) and 100 mg/kg (high dose level). The specific distribution of chloroquine in the ocular tissue

![Figure 1. MS/MS spectrum of chloroquine showing the major fragmentation peak at m/z 247.10 from the precursor ion at m/z 320.19.](image-url)
was first examined using the tissue section from the conventional dose level rat. In this experiment, Matrix-A (see “Experimental” section) was deposited to the tissue using the manual airbrush. The digital photo image of ocular tissue and its MALDI-MS image obtained from an abundance plot of the m/z 247.10 chloroquine fragment ion are shown in Fig. 3. The image of the m/z 247.10 fragment ion derived from chloroquine was weak and the specific distribution to melanin-containing tissue was not observed. In order to obtain the specific distribution image of chloroquine, the tissue section from the high dose level rat was prepared by manual airbrush using Matrix-A and further investigated. Although chloroquine was administered at a high dose level of 100 mg/kg to the pigmented rat, the image of the m/z 247.10 chloroquine fragment ion was again weak and heterogeneously distributed. As a result, the specific distribution of chloroquine to melanin-containing tissue was hardly observed (Fig. 4). There was also a high possibility that this image detected only chloroquine that was not bound to melanin. The specific distribution of chloroquine to melanin-containing tissue at 20 mg/kg or 100 mg/kg dosing was not observed in the application of Matrix-A with the airbrush because melanin within the eye retina has a strong affinity, possibly forming a complex with chloroquine. An improvement of the matrix is required in order to detect the chloroquine strongly associated with melanin. As a result, the acidity and concentration of the organic solvent in the matrix were raised to break the melanin and chloroquine complex.

**Application of Matrix-B using the manual airbrush.** In order to extract chloroquine from melanin-rich tissues (e.g., the retina) into the matrix, a higher concentration of acid and organic solvent is used to prepare Matrix-B (see “Experimental” section) for MALDI-Imaging MS. The MALDI-MS image showing the specific distribution of the m/z 247.10 chloroquine-derived fragment ion in the ocular tissue from
Figure 4. MS/MS (m/z 320.19 → 247.10) image of ocular tissue section in pigmented rat using Matrix-A with the airbrush (left) and digital image of the section (right). The rat was administered at 100 mg/kg and the section was excised at 24 h after administration.

Figure 5. MS/MS (m/z 320.19 → 247.10) image of ocular tissue section in pigmented rat using Matrix-B with the airbrush (left) and digital image of the section (right). The rat was administered at 20 mg/kg and the section was excised at 24 h after administration.

Figure 6. MS/MS spectra for the precursor ions (m/z 320.19) at points 1–6 in Fig. 8.
the pigmented rat administered 20 mg/kg was observed (Fig. 5). The MS/MS spectra of points 1 to 6 in Fig. 5 are shown in Fig. 6. The chloroquine-derived fragment ion at \( m/z \) 247.10 was only observed in the MS/MS spectra of points 1, 2, 3 and 4, but not points 5 and 6. In comparison to Matrix-A, the use of Matrix-B was shown to assist in the generation of stronger MALDI-MS signal intensities and a better image of the distribution of chloroquine.

MALDI-Imaging MS of the \( m/z \) 247.10 region of the MS/MS spectrum of \( m/z \) 320.19 in blank tissue dosed with vehicle only is shown in Fig. 7. Although there was some random noise ions found, no interference with detection of chloroquine was observed.

The MALDI-MS image of the specific distribution of chloroquine within the retina region of the ocular tissue from rats dosed with 20 mg/kg of chloroquine was confirmed using Matrix-B with the airbrush, and found to be similar to the autoradiogram after oral administration of \(^{14}\)C-chloroquine reported previously. However, after running the MALDI imaging experiments using Matrix-B containing 1% TFA, the MALDI-MS sensitivity was observed to decline more rapidly than normal. TFA is a very strong ion-pairing reagent and the rapid decrease in sensitivity suggests contamination of the instrument by TFA in the matrix solvent. Therefore, a comparison between the use of 1% TFA and 5% FA in the matrix solution was also investigated.
**Application of Matrix-C using the manual airbrush.** The image profiles (sensitivity and homogeneity) of the m/z 247.10 chloroquine-derived fragment ion in the ocular tissue of rats administered with 20 mg/kg chloroquine was compared between Matrix-B (containing 1% TFA) and Matrix-C (containing 5% FA, see “Experimental” section). As shown in Fig. 8, the image profile using Matrix-C was approximately equivalent to that using Matrix-B (eye sections were obtained from the same rat). Since high concentrations of TFA (1%) in Matrix-B reduces the sensitivity of the MS instrument more rapidly, it is essential to switch to non-TFA matrices like Matrix-C which, although containing a high concentration of FA (5%), did not cause any MALDI source contamination beyond standard instrument operation. Furthermore, the extracted ion intensity of the m/z 247.10 fragment ion was observed to be nearly two times higher with Matrix-C than with Matrix-B, illustrating sensitivity benefits for the experiment. As a result, it is practical to use Matrix-C over Matrix-B.

**Optimization of matrix application method (manual airbrush vs. automatic sprayer)**

The application of the matrix in MALDI-Imaging MS is one of the most important points to obtaining a reliable and reproducible image of the drug distribution in the tissue. However, the matrix is still popularly applied with an
airbrush and the reproducibility of the images becomes more a factor of the spray operator’s skill. In addition, since the matrix is usually deposited as large heterogeneous droplets, the resultant image of the drug distribution is often varied and shows poor detection limits. As a result the automatic matrix spraying method was investigated for better imaging using the TM-Sprayer from HTX Technologies. Matrix-D (see Experimental section) was used with the TM-Sprayer.

Figure 9 shows the MALDI-MS image of the m/z 247.10 fragment ion derived from the precursor ion at m/z 320.19 in the blank ocular tissue of the vehicle-administered rat that had been prepared using the automated TM-Sprayer with
Matrix-D. The MS/MS spectra of points 1 to 6 in Fig. 9 is shown in Fig. 10 illustrating that the chloroquine-derived fragment ion at m/z 247.10 was not detected. This also highlights the importance of MS/MS in MALDI Imaging MS since a strong possible precursor ion was observed at m/z 320.19 in the spectrum. Furthermore, although the experiments were conducted with the resolution of the instrument set to 10 000 FWHM, it may be possible to obtain better specificity in the images by applying higher resolution settings when available. This increased specificity may be additionally combined with ion mobility separations in mass spectrometers to remove background derived signals from the MALDI-MS image.

The image profile of the m/z 247.10 chloroquine-derived fragment ion in the ocular tissue of rats administered with 20 mg/kg of chloroquine was compared between manual airbrush application of Matrix-C and automated TM-Sprayer application of Matrix-D (Fig. 11). A good MALDI image of the chloroquine distribution was generated by the TM-Sprayer. Although its image profile was essentially identical to that generated by the airbrush, it was found that the automated application of the matrix using the TM-Sprayer resulted in better sensitivity when compared to manual application. This improvement in sensitivity was confirmed by the spectrum peak intensity of the m/z 247.10 chloroquine-derived fragment ion. As shown in Fig. 12, the intensity of the fragment ion obtained using the automated TM-Sprayer was 14 times stronger than that by the manual airbrush.

CONCLUSIONS

The approach of MALDI-Imaging using a Q-TOF type of tandem mass spectrometer suggests that the specific distribution to melanine-containing tissue, retina, of the parent drug in the ocular tissue of pigmented rat administered with a 20 mg/kg dose of chloroquine can visually be obtained with ease by applying an acidic CHCA matrix using an automated sprayer. In addition, this approach does not require the isotope labeling of the analytes or any extraction procedures from the tissues, presenting several advantages over existing technologies used in drug bioanalysis. Therefore, this technology will contribute significantly to elucidating the mechanism of drug efficacy and/or toxicity, and may provide deeper insight into drug discovery and development processes to help reveal the cause of efficacy or side effects often associated with drug administration at the molecular level.

Acknowledgements

The authors would like to thank Jose Castro-Perez, now at Merck Research Laboratories (Rahway, NJ, USA), for cooperation in this research.

REFERENCES