Controlled-pH Tissue Cleanup Protocol for Signal Enhancement of Small Molecule Drugs Analyzed by MALDI-MS Imaging

ARTICLE *in* ANALYTICAL CHEMISTRY · APRIL 2012

Impact Factor: 5.64 · DOI: 10.1021/ac203322q · Source: PubMed

analytical chemistry

Technical Note

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Controlled-pH Tissue Cleanup Protocol for Signal Enhancement of Small Molecule Drugs Analyzed by MALDI-MS Imaging

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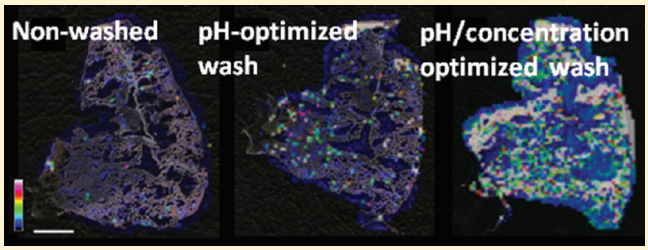
ABSTRACT: The limit of detection of low-molecular weight compounds in tissue sections, analyzed by matrix assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI), was significantly improved by employing sample washing using a pH-controlled buffer solution. The pH of the washing solutions were set at values whereby the target analytes would have low solubility. Washing the tissue sections in the buffered solution resulted in removal of endogenous soluble ionization-suppressing compounds and salts, while the target compound remained in situ with minor or no delocalization during the buffered washing procedure. Two pharmaceutical compounds (cimetidine and imipramine) and one new protease inhibitor compound were successfully used to evaluate the feasibility of the pH-controlled tissue washing protocol for MALDI-MSI. Enhancement in signal-to-noise ratio was achieved by a factor of up to 10.



Matrix assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI) has recently become an established technique that enables the mapping of molecular species directly from tissue sections.[1−3] The technique produces qualitative and relative quantitative information about the localization of diverse compounds ranging from small molecule drugs,[4] lipids,[5] and peptides,[6] to proteins[4,7] in an increasing variety of samples/tissues.[6,8,9] In addition to the ability to collect distribution data, we have recently demonstrated that, through the deposition of quantitation standards on vehicle control tissue sections, quantitation of the distribution of drugs administered in vivo at pharmacological dose is possible by MALDI-MSI.[10] While MALDI-MSI can offer both high spatial resolution (<10 μm)[11] and high mass spectral resolution (>1 000 000)[12] directly from tissue samples, a major limiting constraint for the application of the technology is that sensitivity can be hampered significantly by endogenous compounds such as salts and other ionization suppressing species present within the tissue sample.[13] Lipid species, for example, interfere with the MALDI matrix crystallization process and thus may suppress the signal. Signal suppression is often so pronounced that no detection of target analyte is possible even when dosing is administered substantially higher than required as determined by off-tissue analysis. To date, a limited number of sample preparation protocols have been described that enhance the sensitivity of detection of proteins and peptides analyzed by MSI. Such methods involve sample washing prior to MALDI matrix application and subsequent analysis. Typically, an aqueous/ethanol solution is used to bath the samples for 30 to 60 s and enables salts, lipids, and cell debris to be washed from the tissue section. A second washing stage, performed in 100% ethanol, is used to dehydrate and fix the tissue.[14−16] Such sample preparation methods rely on the low solubility of many of the peptides in the organic solutions and hence limit loss of target analytes or their delocalization. However, in spite of the efficiency of these protocols for MALDI-MSI at the proteomic level, they are not suitable for small molecule and pharmaceutical drug analysis due to the substantial/total loss of such compounds in the organic solvents resulting in significant analyte delocalization and often complete removal from the sample.

We describe here the use of a sample washing protocol that enables improved detection of small molecule drugs when analyzing by MALDI-MSI. Our protocol employs an aqueous buffered wash solution with the pH adjusted to a point whereby the target analyte is insoluble. Subsequent bathing of the sample section

Received: December 14, 2011
Accepted: April 16, 2012
Published: April 16, 2012

   dx.doi.org/10.1021/ac203322q | *Anal. Chem.* 2012, 84, 4603−4607

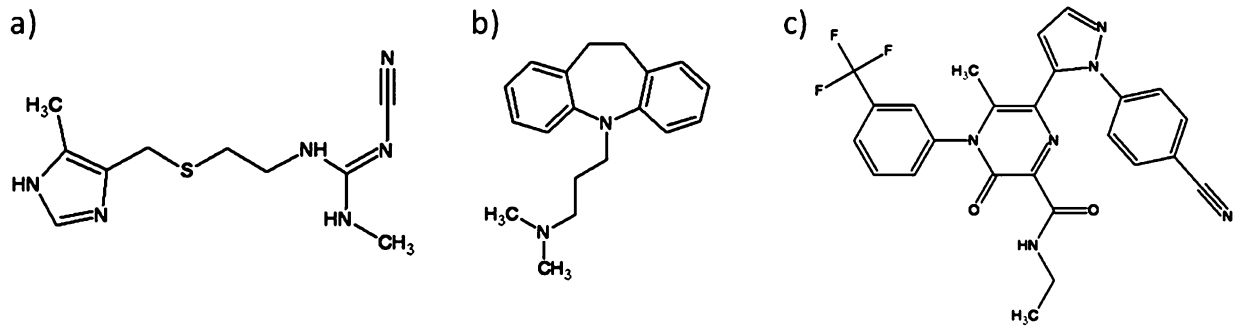


Figure 1. Structures of the model compounds used to evaluate pH-controlled tissue cleanup protocol: (a) cimetidine, (b) imipramine and (c) compound C.

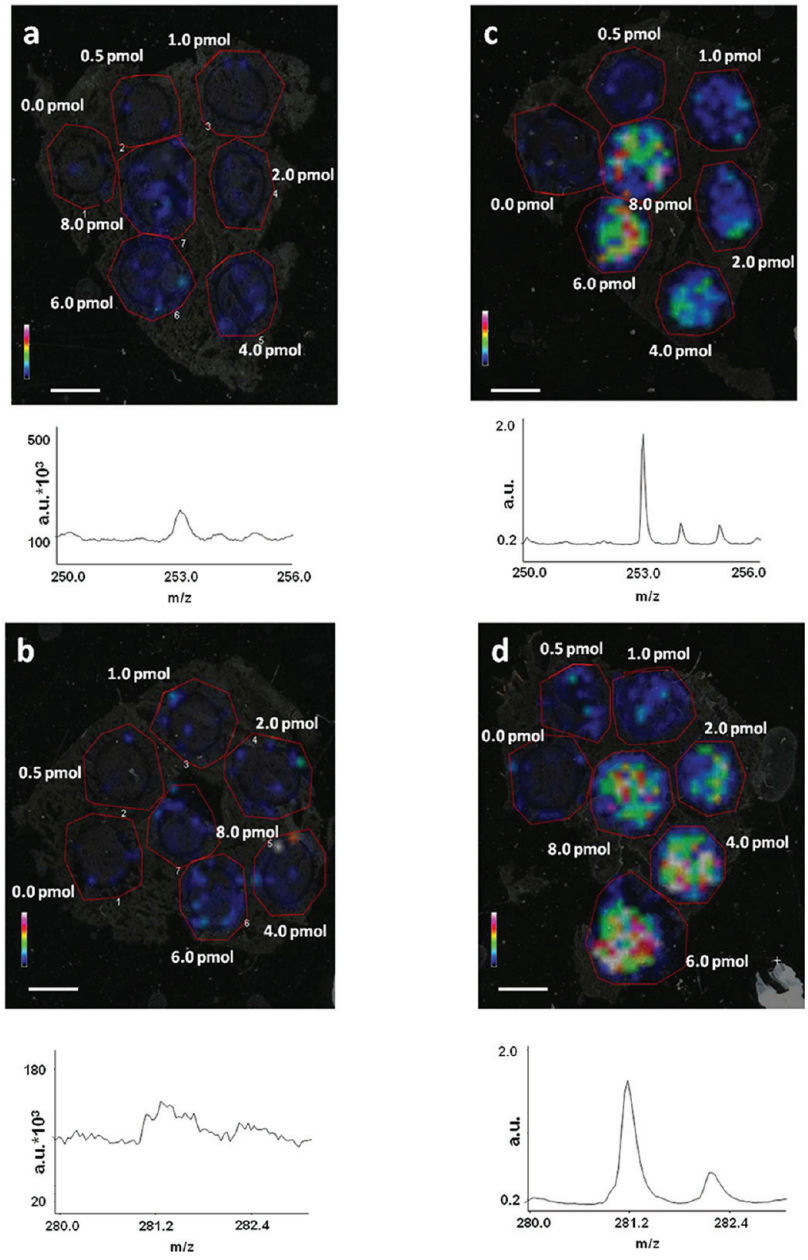


Figure 2. MALDI-MSI relative abundance and distribution of cimetidine (m/z 253.1) and imipramine (m/z 281.1) following manual spotting of standard solutions onto tissue sections (0.2 μL, 0–8.0 pmol) overlaid on an optical image, with corresponding summed spectra of all concentrations. (a, b) Nonwashed tissue sections and (c, d) tissue sections washed in pH buffered solution. The corresponding summed spectra showed 8- and 10-fold increase in signal-to-noise for cimetidine and imipramine, respectively. (Data displayed in rainbow scale over the same range, scale bar 2 mm).

dx.doi.org/10.1021/ac203322q | *Anal. Chem.* 2012, 84, 4603–4607

(thaw-mouthed on MALDI target) enables the removal of the soluble endogenous salts and other suppressant compounds from the tissue section with minimal delocalization of the compounds of interest. Such a sample preparation methodology is applicable to researchers wanting to analyze the abundance and distribution of small molecule drugs by MALDI-MSI. Cimetidine, imipramine, and compound C, a proprietary protease inhibitor, were used as model compounds for this study (Figure 1).

EXPERIMENTAL SECTION

Chemicals. Ammonium acetate and ammonium hydroxide were purchased from Fluka Chemie (Buchs, Switzerland). Water, methanol, and trifluoroacetic acid (TFA) were from Merck (Darmstadt, Germany). Cimetidine and imipramine were obtained from Sigma–Aldrich (St. Louis, MO, USA). Compound C (6-[2-(4-cyanophenyl)pyrazol-3-yl]-*N*-ethyl-5-methyl-3-oxo-4-[3-(trifluoromethyl)phenyl]pyrazine-2-carboxamide) was provided by AstraZeneca R&D (Lund, Sweden).

Tissue Preparation. Animal experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) approved by the ethical Committees on Animal Experiments in Lund/Malmö, Sweden (no. M84-05) and at Karolinska Institutet, Stockholm, Sweden (no. N351/08). Adult Male Wistar rats weighing approximately 350 g were exposed to the aerosol of a nebulized solution of compound C in an in-house built Battelle Design, two-stage nose-only flow-past inhalation chamber.[17] Control and dosed animals were anesthetized and sacrificed 15 min after administration. The lungs were rapidly dissected out and immediately frozen in dry ice-cooled isopentane. The lungs were stored at −80 °C until required for sectioning. C57Bl/6 male mice, 3 months old, were group-housed in air-conditioned rooms (12-h dark/light cycle) at 20 °C with humidity of 53%. Animals were administered with single doses of imipramine, 10 or 30 mg/kg by intraperitoneal (i.p.) injections. Mice were euthanized 30 min after injection, and their brains were rapidly removed, frozen in dry ice-cooled isopentane, and stored at −80 °C. The frozen lung and brain tissues were cut by cryostat-microtome (Leica CM3050S, Leica Microsystems, Germany) at a thickness of 15 to 20 μm. Lung sections were taken from the long flat frontal plane of the left lobe, providing sections of the central airways. Sagittal brain sections were cut at a thickness of 12 μm. Tissue sections were transferred by thaw mounting onto conductive indium tin oxide (ITO) glass slides (Bruker Daltonics, Bremen, Germany), prior to storage at −80 °C. Sections were desiccated at room temperature for 15 min prior to the sample washing protocol and MALDI matrix application.

MALDI-MSI Analysis. Cimetidine and imipramine solutions (2.5–40 μM in 50% methanol, 0.2% TFA) were manually spotted on vehicle control tissue sections (0.2 μL resulting in 0.5–8 pmol/spot). The predicted molar solubility of the drugs in different pH values were used, and the pH of ammonium acetate buffer was adjusted to the point of lowest solubility for each of the drugs of interest.[18] Tissue sections from animals administered with drug and with drug spotted onto the tissue (vehicle control) were washed by total immersion in pH-buffered washing solution for 10 s, followed by 20 s of spinning using an in-house constructed slide spinner to quickly remove the solvent droplets from the surface, prior to complete drying under high vacuum for 15 min. MALDI matrix, (2,5-dihydroxybenzoic acid, 30 mg/mL, 50% methanol, 0.2% TFA) was applied by an automatic matrix sprayer (ImagePrep, Bruker Daltonics). MALDI-MSI was performed using an Ultraflex II TOF/TOF (Bruker Daltonics) in positive ion reflectron mode using a Smartbeam II 200 Hz laser. The mass spectrometer parameters were adjusted for optimal imaging performance as follows: ion source 1, 24.9 kV; ion source 2, 21.8 kV; lens voltage, 11.03 kV; gain factor, 13.7×; matrix suppression mass cutoff, 150 Da. Laser spot size was set at medium focus (~50 μm laser spot diameter), and laser power was optimized at the start of each run and then fixed for the MALDI-MSI experiment while all the comparative images were acquired with the same laser intensity. Tissue sections were analyzed in a random order to prevent any possible bias due to such factors as matrix degradation or variation in mass spectrometer sensitivity. MSI data was analyzed and normalized using FlexImaging version 2.0 (Bruker Daltonics). Regions of interest were manually defined in the analysis software using both the optical image and MSI data image where the spatial resolution was also defined (typically 200 μm). Masses were selected with a mass precision of ±0.1 Da.

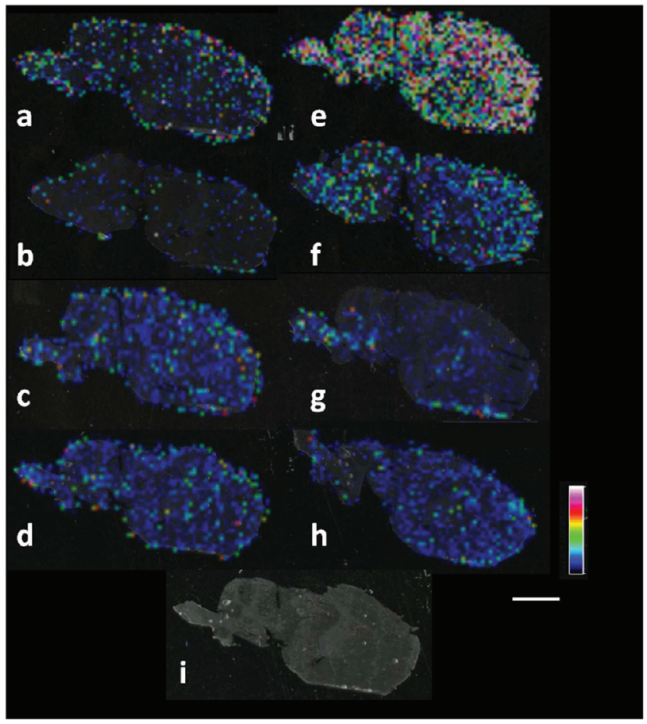


Figure 3. MALDI-MSI relative abundance and distribution of imipramine (m/z 281.1) in sagittal mouse brain tissue sections. Imipramine was administered by in vivo i.p. injections. Images (a–d) correspond to nonwashed tissue sections, while tissue sections (e–h) are treated by different washing solutions. (a, b) 30 and 10 mg/kg, respectively, nonwashed tissue sections and (e, f) washed by the buffer at pH 10. (c) 10 mg/kg nonwashed tissue section and (g) tissue section washed by the buffer at pH 3. (d) 10 mg/kg nonwashed tissue section and (h) washed by nonbuffered water. (i) The representative optical image of sagittal mouse brain tissue section. The results show that a significant increase in signal-to-noise was achieved when the buffer solution was pH adjusted to a point where imipramine has low solubility, which enables the removal of suppressant molecular species from the tissue (e, f), whereas no signal enhancements were achieved when acidic or nonbuffered solutions were used (g, h). (Data displayed in rainbow scale over the same range, scale bar 2 mm.) The identity of imipramine was verified by tandem MS (MS/MS) directly on the tissue sections, and the product ion spectra were compared with those obtained from imipramine standard (data not shown).

dx.doi.org/10.1021/ac203322q | *Anal. Chem.* 2012, 84, 4603–4607

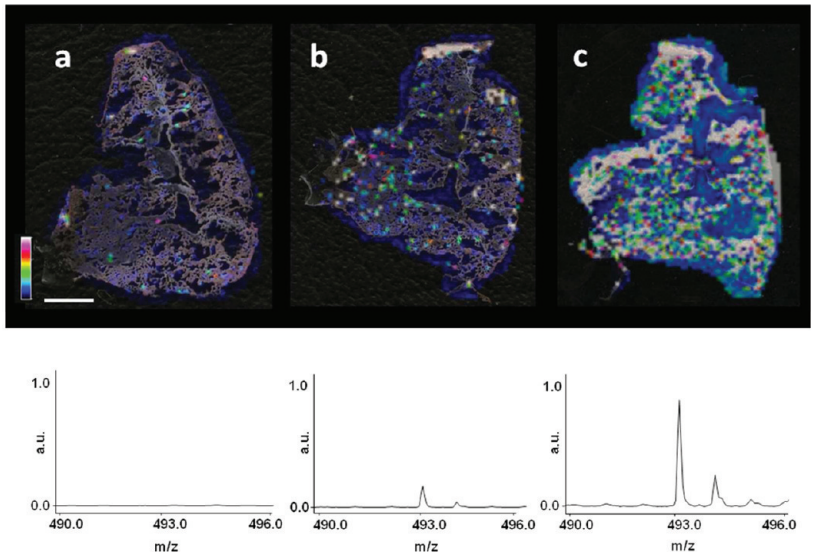


Figure 4. MALDI-MSI relative abundance and distribution of compound C (m/z 493.2) in lung tissue sections. Compound C was administered to rats by in vivo inhalation. Corresponding summed spectra of the whole tissue sections are shown below the images. (a) Nonwashed tissue section; tissue section washed (b) in 10 mM ammonium acetate at pH 6 and (c) in 100 mM ammonium acetate solution at pH 6. Increased signal-to-noise was achieved when a higher concentration of the buffer solution was used. (Data displayed in rainbow scale over the same range, scale bar 2 mm.) The identity of compound C was determined by the m/z ratio. No detection of the compound was obtained on the control tissue, which was analyzed at the same time as the dosed tissue.

RESULTS AND DISCUSSION

Vehicle control lung tissue sections were spotted with known concentrations of cimetidine and imipramine to evaluate the efficacy of the buffered-wash solution protocol. Cimetidine and imipramine both have basic functional groups which are neutralized at higher pH. For cimetidine, solubility at pH 1 is 2.16 M but at pH 10 is 2.9 mM. For imipramine, solubility at pH 1 is 0.23 M but at pH 10 is 57 μM. Therefore, using a buffered wash solution at pH 10, it is predicted that for cimetidine and imipramine there will be an improvement in detection as well as limited spatial delocalization at a pH where the drugs have low solubility. Figure 2 shows MALDI-MSI data obtained for different concentrations of cimetidine and imipramine deposited onto tissues (with and without subsequent processing with buffered wash solution). The limits of detection were improved by the factors of 10 for imipramine and 8 for cimetidine when the tissues were treated. Also demonstrated is that no or minor delocalization of the drug occurs; however, delocalization and/or removal of the drugs occurs when either ammonium acetate with an acidic pH or water were used as washing solutions. The tissue sections from in vivo drug administered animals (compound C, rat lung; imipramine, mouse brain) were analyzed to evaluate the applicability of the buffered-wash solution protocol. Figure 3 is a summary of the MSI data collected using the imipramine post-treatment brain tissue sections. Again, following the buffered-washing protocol, the overall signal-to-noise for the protonated imipramine was shown to be considerably enhanced, with the limit of detection improved by the factor of approximately 3. The effect of treating of the brain tissues with buffer solution with acidic pH as well as nonbuffered water is also presented in Figure 3. Both the delocalization and decrement of signal-to-noise correlate with the solubility of imipramine being greater in acidic conditions compared to when in neutral or alkaline conditions. Our washing protocol was also employed on tissue sections taken following an in vivo lung treatment using compound C. Tissue sections were washed with either 10 or 100 mM of ammonium acetate, and the results are summarized in Figure 4. Compound C is a lipophilic compound that has low solubility in aqueous solutions. Using our buffered sample processing method, significant improvement in the signal-to-noise ratio for detection of compound C was achieved when 100 mM of ammonium acetate was adjusted to pH 6. More improvement in signal when the 100 mM ammonium acetate (compare to 10 mM ammonium acetate) wash solution is used can be attributed to the greater proportion of ammonium acetate ions that can be exchanged for endogenous ions that can cause suppression during MSI analysis of the target compound. It should be noted that when our described protocol is used for highly basic compounds, which would require a highly alkaline buffered wash solution, the resulting alkaline tissue surface could hamper the formation of protonated species. However, this might well be overcome using a higher proportion of TFA in the matrix solution.

CONCLUSION

In the data presented here, we have shown that tissue preparation involving a buffer solution with the pH adjusted to a point where the target analyte has low solubility enables the removal of suppressant molecular species from the tissue that results in an improved signal-to-noise ratio for the target analytes when analyzed by MALDI-MSI. Furthermore, this improvement in signal-to-noise is achieved without significant spatial delocalization of the target compound during the washing procedure. We also demonstrate that a higher concentration buffered solution results in a greater improvement in signal-to-noise enhancement, and we propose that this is probably due to higher yield of an ion exchange process between suppressant and buffer ions. It is worth noting that the reported protocol is limited to targeted MALDI-MSI analysis; however, for detection of unknown compounds, a series of experiments using different pH values could be considered. A fuller statistical evaluation of the effect of pH-buffer washing of tissues analyzed by MSI will be required to better understand the effectiveness

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of the described protocol. However, such comparisons for MSI data are still to be resolved, but we show that the comparative images presented here provide a strong case for the implementation of the protocol to active research projects. We believe that the described wash protocol is easily implemented within any standard MSI workflow and will be of substantial benefit for MALDI-MSI researchers working within the pharmaceutical and metabolomics fields.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors acknowledge Anna Nilsson, Nadine Mascini, and Sara Ståhl for their guidance and contribution to this work. This work is financially supported by Swedish Research Council under grant numbers 2008-5597, 2009-6050, 2010-5421, and 2010-0881 and the Knut and Alice Wallenberg Foundation.

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