**REPORT DATE** (DD-MM-YYYY) | **REPORT TYPE** | **DATES COVERED** (From - To)
---|---|---
2000 | Open Literature |

**TITLE AND SUBTITLE**
MALDI-ToF/MS as a Diagnostic Tool for the Confirmation Of Sulfur Mustard Exposure

**AUTHOR(S)**
Price, EO, Smith, JR, Clark, CR, Schlager, JJ, and Shih, ML

**PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-UV-PA
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

**SPONSOR/MONITOR’S ACRONYM(S)**
US Army Medical Research
Anniston Army Depot
Aberdeen Proving Ground, MD 21010-5400
ATTN: MCMR-UV-PA

**ABSTRACT**
See reprint.

**SUPPORTING NOTES**
Published in a special issue of Journal of Applied Toxicology, 20, S193-S197, 2000.

**SUBJECT TERMS**
sulfur mustard, laser desorption, mass spectrometry, chemical agents, diagnosis, protein adducts

**DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; distribution unlimited

**NUMBER OF PAGES**
5

**NAME OF RESPONSIBLE PERSON**
John R. Smith
410-436-1728

**SECURITY CLASSIFICATION OF:**
a. REPORT | b. ABSTRACT | c. THIS PAGE
---|---|---
UNCLASSIFIED | UNCLASSIFIED | UNCLASSIFIED

**LIMITATION OF ABSTRACT**
UNLIMITED

**NUMBER OF PAGES**
5

**TELEPHONE NUMBER**
410-436-1728
MALDI-ToF/MS as a Diagnostic Tool for the Confirmation of Sulfur Mustard Exposure†**

Elvis O. Price, J. Richard Smith, Connie R. Clark, John J. Schlager and Ming L. Shih*
Pharmacology Division, Applied Pharmacology Branch, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5425, USA

Key words: laser desorption; mass spectrometry; chemical agents; diagnosis; sulfur mustard; protein adducts.

The continual threat of chemical and biological warfare has prompted the need for unambiguous analytical methods for the confirmation of agent exposure. In this paper, we have investigated the use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF/MS) as a diagnostic tool for this purpose. Mass spectral studies of the interaction of sulfur mustard (bis-(2-chloroethyl) sulfide, HD) with hemoglobin and metallothioneine were conducted. In vitro experiments with purified proteins were performed, using both HD and chloroethylsulfide (CEES), in an effort to determine the extent of alkylation and occurrence of HD cross-linking using the MALDI-ToF/MS technique.

In a typical experiment, 50 ml of 5 mM HD in acetonitrile was added to an equal volume of 0.5 mM hemoglobin in deionized water followed by vortexing and incubation at room temperature. After 24 h, the samples were analyzed by MALDI-ToF/MS. Mass spectral results indicated the presence of at least two distinct alkylation adducts for both HD and CEES experiments. These results demonstrate that MALDI-ToF/MS is a useful analytical technique to investigate the interaction of HD with biomolecules and may be employed potentially as a diagnostic tool for the confirmation of exposure to chemical warfare agents. Published in 2000 by John Wiley & Sons, Ltd.

INTRODUCTION

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF/MS) is a relatively new mass spectrometric technique that has been used in molecular weight determination as well as mixture analysis of biomolecules such as proteins and peptides, nucleotides, glycolipids, carbohydrates and oligosaccharides. This technique has been proven to be a comparatively advantageous analytical technique that allows for fast mass analysis time and requires little sample preparation of biomacromolecules. In addition, MALDI-ToF/MS exhibits high sensitivity (detection limits < subpicomolar), good mass accuracy (<0.05%), large detection range (above 100 kDa) and high resolution capabilities. Initially, a sample is prepared by mixing the saturated matrix solution (usually prepared with pure forms of α-cyano-4-hydroxycinnamic acid, 3,5-dimethoxy-4-hydroxycinnamic acid or 2,5-dihydroxy benzoic acid in 0.3% aqueous trifluoroacetic acid containing 30% acetonitrile) with the analyte solution at an experimentally determined ratio before 1–2 μl of the final mixture is placed on the sample probe. The ‘spotted’ sample is then allowed to dry either under vacuum or in air until the analyte and matrix molecules co-crystallize on the probe. Subsequently, the probe is placed into the MALDI-ToF mass spectrometer where the matrix is desorbed by an ultraviolet (UV) laser beam permitting the analyte to be ionized, through matrix ionization energy transfer, predominantly as intact quasimolecular ions. The generated ions are then accelerated down a TOF path where they are separated based on their relative mass-to-charge ratios (m/z). The ions are then detected at the end of the flight tube by the mass detector while a real-time mass spectrum is observed simultaneously on a computer monitor.

Studies have shown previously that MALDI-ToF/MS can be utilized as an effective and rapid means for determining and identifying site modifications of proteins by alkylating reagents. Because it has been reported that chemical agents such as nerve agents and sulfur mustard (HD) form covalent linkages with various cellular macromolecules, these covalent interactions can conceivably be monitored and/or specifically identified as potential biological markers of exposure rather easily using MALDI-ToF/MS. Our preliminary studies are focused on the identification of the HD adducts of hemoglobin (Hb) and metallothioneine (MT). Interpretation of the mass spectra was attempted to identify the structures of the HD–biopolymer adducts.

* Correspondence to: M. L. Shih, Pharmacological Division, Applied Pharmacology Branch, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5425, USA.
† This article is a U.S. Government work and is in the public domain in the U.S.A.
** The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army of the Department of Defense.

Published in 2000 by John Wiley & Sons, Ltd.
Figure 1. The MALDI mass spectra of Hb before (a) and after (b) sulfur mustard exposure.

EXPERIMENTAL

Instrumentation and sample preparation
The MALDI mass spectra were obtained in positive mode using a linear ToF instrument equipped with time-lag focusing (Voyager-DE Biospectrometry Workstation, PerSeptive Biosystems, Inc., Framingham, MA). Ionization was accomplished with a nitrogen laser operating at wavelength 337 nm with a 3-ns pulse width, and generated ions were accelerated at 20 and 25 kV. The matrix was prepared as a saturated solution of α-cyano-4-hydroxycinnamic acid (α-CHCA) or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in deionized water–acetonitrile (7:3) containing 0.3% trifluoroacetic acid (TFA). All protein samples, before and/or after exposures, were diluted to at least 5 μM concentration before MALDI/MS analysis. Approximately 1 μl of α-CHCA solution was placed on the MALDI sample probe, and 1 μl of the diluted MT sample(s) was immediately added on top of the matrix droplet. The resulting sample–matrix droplet was then allowed to dry in air until complete co-crystallization occurred. The Hb samples were prepared for MALDI mass analysis in the same manner except that the α-CHCA matrix was replaced with sinapinic acid matrix.

Table 1. Proposed Hb–HD alkylated products observed by MALDI-ToF/MS (data from Fig. 1b)

<table>
<thead>
<tr>
<th>Proposed type of HD–Hb adduct</th>
<th>Total mass of HD moieties</th>
<th>Number of HD units adducted to Hb</th>
<th>Peaks observed (m/z)</th>
<th>Calculated mass (relative to m/z 15122.0, unreacted Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOH</td>
<td>105.2</td>
<td>1</td>
<td>15226.2</td>
<td>15227.2</td>
</tr>
<tr>
<td>M, MOH</td>
<td>193.4</td>
<td>2</td>
<td>15314.0</td>
<td>15315.4</td>
</tr>
<tr>
<td>M, 2MOH or 2M, MCI</td>
<td>299.6 or 300.0</td>
<td>3</td>
<td>15418.7</td>
<td>15420.6 or 15422.0</td>
</tr>
<tr>
<td>M, 2MOH, MCI or 2M, 2MCI</td>
<td>422.2 or 423.6</td>
<td>4</td>
<td>15540.9</td>
<td>15544.2 or 15545.6</td>
</tr>
</tbody>
</table>

M = -CH₂CH₂SCH₂CH₂; MOH = -CH₂CH₂SCH₂CH₂OH; MCI = -CH₂CH₂SCH₂CH₂Cl.
MALDI-ToF/MS TO DETECT HD EXPOSURE

Figure 2. The MALDI mass spectra of MT before (a) and after (b) sulfur mustard exposure.

Chemicals
Rabbit liver MT, human Hb, chloroethylethylsulfide (CEES), α-CHCA, sinapinic acid, TFA and acetonitrile (HPLC grade) were obtained from Sigma Chemical Company (St Louis, MO). Sulfur mustard was obtained from the US Army Edgewood Research, Development and Engineering Center (Aberdeen Proving Ground, MD) in 97.5% purity.

Exposure of Hb to HD
Hemoglobin samples of 50 μL each of 3 mM, 0.5 mM and 0.025 mM were prepared in deionized water. Subsequently, 50 μL of 5 mM HD in acetonitrile was added to each sample, followed by vortexing. The three samples were allowed to incubate at room temperature for at least 24 h before they were subjected to MALDI/MS analysis.

Exposure of MT to HD
Aqueous MT samples of 0.5 mM (2 μL), 0.5 mM (4 μL) and 0.5 mM (8 μL) were each exposed to 2 μL of 4 mM HD prepared in acetonitrile. Samples were allowed to incubate for 24 h at room temperature before MALDI/MS analysis was performed.

Exposure of Hb to CEES
The CEES–Hb experiments were modeled after the HD–Hb experiments except that 10 mM CEES was substituted for HD.

RESULTS AND DISCUSSION
Alkylated products were observed when Hb and MT were exposed to HD. Figure 1 depicts the MALDI-ToF/MS spectra of Hb before and after HD exposure. The peaks in Fig. 1a at m/z 15126.4 and m/z 15868.2 correspond to the singly protonated ([M+H]+) α- and β-subunits of native human Hb (control), respectively. These experimental mass values closely compare to the calculated mass values of the α- and β-subunits (15127 and 15868 Da) of Hb. Figure 1b represents the HD-exposed Hb where the ion at m/z 15122.0 corresponds to the unalkylated Hb, and m/z 15226.2 corresponds to Hb with one adducted HD molecule with a hydroxyl functionality at the free end of the HD moiety. The additional peaks observed at higher mass shifts for all HD–Hb experiments are attributed to two or more alkylation events of the various possible types.

Published in 2000 by John Wiley & Sons, Ltd.

of HD moieties added to Hb (Table 1). Commercial rabbit MT produced an [M+H]^+ ion at m/z 6125.9 (Fig. 2a), representing the major isoform of MT. Figure 2b of the HD-exposed MT displays several peaks that are attributed to HD alkylation products of MT and the unreacted MT. Table 2 lists the m/z values and the proposed structures of these HD-MT adducts. Both MT and Hb were incubated with CEES to eliminate concerns of HD self-reaction. Based on the chemical structure of CEES (CICH₂CH₂SCH₂CH₃), it was expected that the CEES molecule would add to MT and Hb in only one possible manner. The mass spectra of the alkylated products of the Hb-CEES and MT-CEES experiments are shown in Figs 3 and 4. The peaks that correspond to the CEES alkylated species of Hb and MT are well-defined single peaks that are separated from each other by ca. 89 ± 1.5 Da, reflecting the molecular weight addition of a single CEES molecule. As the concentration of HD or CEES was increased, while maintaining constant protein concentration, an increase in alkylation species was observed (data not shown).

**CONCLUSION**

Investigation of the interaction of HD with Hb and MT in vitro by way of MALDI-ToF/MS analysis is
We have demonstrated clearly that MALDI-ToF/MS can be utilized to study the in vitro interaction of HD with cellular macromolecules and, given the attractive characteristics of MALDI-ToF/MS, this technique may be employed potentially as a diagnostic tool for the confirmation of chemical agent exposure.

REFERENCES