

RESEARCH ARTICLE

# Methylhexahydrophthalic anhydride adducted albumin tryptic peptides in nasal lavage fluid

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## Abstract

Methylhexahydrophthalic anhydride (MHHPA) is a reactive, low molecular weight chemical used in products such as plastics, paints, and electronic components. Exposure to MHHPA may lead to work-related airway diseases such as rhinitis, conjunctivitis, and asthma. Twelve subjects employed at a plant manufacturing electrical capacitors using MHHPA were included in this study. Nasal lavages were collected from subjects before work Monday morning and after work Tuesday afternoon. The levels of MHHPA adducted to serum albumin were analyzed with a straightforward work-up method. The samples were trypsinated before being analyzed with a liquid chromatography–triple quadrupole mass spectrometer. The mass spectrometer was run using selected reaction monitoring for six adducted peptides. Also, some biomarkers of effect (albumin, total protein, eosinophil cationic protein, and trypsinase) were analyzed in nasal lavages. Furthermore, the metabolite MHHP acid in urine after work on Tuesday was analyzed by gas chromatography–mass spectrometry. Symptoms from the airways and the eyes and sensitization were registered. The main result of this study is that protein adducts can be analyzed in vivo after low occupational exposures to MHHPA. The results also show a correlation between adducted peptides and albumin in nasal lavage. Furthermore, there may be a difference in the potential to induce hyperresponsiveness between adducts bound to different amino acids.

**Keywords:** Methylhexahydrophthalic anhydride; tryptic peptides; biomarkers; adducts; nasal lavage fluids; mass spectrometry; human serum albumin

## Introduction

Organic acid anhydrides (OAAs) are a group of reactive, low molecular weight compounds that are used in products such as plastics, paints, and electronic components. Exposure to OAAs can lead to an immunoglobulin E (IgE)-mediated allergic reaction with symptoms such as asthma, rhinitis, and conjunctivitis (Venables, 1989). It has been shown in several studies that workers exposed to OAAs may develop airway symptoms with prevalences higher than 60% (Yokota, 1997).

Methylhexahydrophthalic anhydride (MHHPA) and hexahydrophthalic anhydride (HHPA) belong to the group of OAAs. Both of these OAAs are known to be highly sensitizing (Nielsen et al., 2001; Rosqvist et al., 2003; Welinder et al., 1994, 2001), even at exposure levels below 10  $\mu\text{g}/\text{m}^3$ . In addition, these OAAs induce airway diseases at these extremely low exposure levels in a large fraction of exposed

workers (Nielsen et al., 2001, 2006; Rosqvist et al., 2003). It has been suggested that MHHPA may be a more sensitizing compound than HHPA (Rosqvist et al., 2003), but this has not been proven in animal studies (Welinder et al., 1995; Zhang et al., 1998).

In the body, MHHPA is hydrolyzed to methylhexahydrophthalic acid (MHHP acid), and methods for biological monitoring of MHHPA using MHHP acid in urine have been described by Lindh et al. (1997). There is a close correlation between air levels of MHHPA and urinary creatinine-adjusted levels of MHHP acid. In addition, MHHPA reacts and forms adducts with endogenous proteins, e.g. hemoglobin (Hb) (Lindh & Jönsson, 1998a, 1998b) and human serum albumin (HSA) (Rosqvist et al., 2000). It has been shown that the MHHPA adducts of the plasma proteins are highly suitable biomarkers of long-term exposure to MHHPA (Rosqvist et al., 2001). An interesting observation is that MHHPA seems

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to form three times more adducts at the same air levels than does HHPA (Rosqvist et al., 2001). Zhang et al. (1998) showed that it is possible to sensitize animals via conjugates between OAAs and HSA. Thus, these conjugates are of biological relevance.

Kristiansson et al. (2003) characterized the binding of HHPA to HSA in vitro and found the same binding pattern to HSA in nasal lavages (NAL) from an experimentally HHPA-exposed volunteer. In a later paper, Kristiansson et al. (2004) described close correlations between the air levels of HHPA generated in an exposure chamber and the levels of HHPA-adducted tryptic peptides from HSA of five experimentally exposed volunteers. However, the slopes in the correlation between air levels and adduct levels from the subjects were different, indicating an individual difference in formation of the adducts.

It has been suggested that reactions occurring in the nasal mucosa are important, it being the first place of contact with vaporized sensitizing compounds. In fact, it has been suggested that IgE antibodies can be synthesized by B-cells in the nasal epithelium (Durham et al., 1997). Moreover, in HHPA- and MHHPA-exposed workers, symptoms have been reported mainly from the upper airways (Nielsen et al., 2001; Rosqvist et al., 2003). Lindh et al. (1999) showed that rats and guinea pigs exposed to HHPA bound this OAA extensively in the nasal epithelium, and there is a large similarity in the adducts formed by HHPA and MHHPA (Rosqvist et al., 2001). Thus, determination of the levels of MHHPA-adducted tryptic peptides of HSA in NAL possibly reflect the deposition at the target site. Such biomarkers estimate the risk of health effects better than those of internal dose only.

The aim of the present study is to investigate whether MHHPA-HSA peptides can be measured in NAL. Also, we hypothesize that there might be a relationship between the individual levels of MHHPA-HSA peptides and the risk of hyperresponsiveness and airway disease. Furthermore, there might be a difference in the potential to induce hyperresponsiveness between adducts binding to different amino acids. Thus, in this small pilot study, we investigated whether it was possible to analyze MHHPA adducted, tryptic peptides in NAL. We also investigated whether there seemed to be any differences in the associations between different MHHPA adducted HSA peptides, on the one hand, and health effects and data on biomarkers of effect in workers exposed to MHHPA, on the other.

## Materials and methods

### Study population

Employees at a plant manufacturing electrical capacitors using MHHPA went through a medical examination, including a structured interview concerning symptoms from eyes and airways in the past 12 months and ever, smoking habits, and atopy (Nielsen et al., 2001). Furthermore, a common medical and occupational history was obtained. All examinations were made by the same physician (JN), a specialist in occupational medicine. Questions about symptoms were

asked regarding the following: from the eyes: lacrimation, itching, scratching, or burning; from the nose: blocking, itching, running, attacks of sneezing, or bleeding; from the lower airways: attacks of dyspnea, wheezing and/or chest tightness, or dry cough. Symptoms were considered work-related if they appeared during a specific work task or if they improved during weekends or holidays. Furthermore, a physical examination including lung auscultation and a venous blood sample were carried out. The blood sample was later analyzed for specific antibodies of IgE class against HHPA (HHPA-IgE) using ImmunoCAP methodology (Phadia, Uppsala, Sweden).

From these employees, 12 subjects were included in the study. Six subjects sensitized to MHHPA and six non-sensitized subjects were selected. They were all subjected to low levels of exposure to MHHPA in their work. The main exposure was through inhalation of MHHPA vapor. All of the workers had been exposed at least 4 months prior to the start of the investigation. The first sample collection was performed on a Monday morning after a weekend free of exposure. At the end of the study period, Tuesday afternoon, a questionnaire was answered by all the participants about work-related symptoms, number of cigarettes smoked, and work tasks during the observation time. Exposure during two consecutive days was considered to be sufficient to obtain measurable levels of adducts and health effects. Three females and nine males were included in this study. Their age ranged from 30 to 52 years with a median of 37 years. Three of the male subjects had been smoking during the last 2 days. All subjects tested negative for atopy, defined as a positive skin prick test to 12 common allergens (ALK Laboratories, Copenhagen, Denmark). Permission for the study was obtained from the Ethics Committee at Lund University, and all participating workers were informed and gave their written consent.

### Sample collection

NAL were collected from 12 subjects on Monday before their shift started and on Tuesday after their shift ended. Collection of NAL was performed by flushing 0.9% NaCl (15 ml) three times through the nasal cavity. The samples were centrifuged to remove mucous and frozen immediately. Urine samples were collected on Tuesday during the last 4 h of their shift.

### Determination of albumin, eosinophil cationic protein, tryptase, and total protein

The albumin concentration was determined using an albumin enzyme-linked immunosorbent assay (ELISA) kit (Immun Diagnostik, Bensheim, Germany). The concentrations of eosinophil cationic protein (ECP) and tryptase were determined by fluoro-enzyme-immunoassay (ImmunoCAP; Phadia) and total protein concentration using a BCA™ protein assay kit (Pierce, Rockford, IL).

### MHHP acid in urine

MHHP acid in urine was analyzed as described by Jönsson and Lindh (1996). Briefly, the urine was extracted using C18 Bond Elut columns, and the eluate was derivatized with

pentafluorobenzyl and analyzed by gas chromatography-mass spectrometry (VG Trio 1000MS; Fisons, Manchester, UK, coupled to a Carlo-Erba 8065 GC equipped with an A200S auto-sampler; Carlo-Erba, Milan, Italy). The creatinine concentrations were analyzed according to an enzymatic method described by Mazzachi et al. (2000).

### *In vitro synthesis of HSA-MHHPA conjugates*

Fifty milligrams of HSA were dissolved in 4 ml of phosphate buffered saline (PBS) containing ethyldiaminetetraacetic acid (EDTA). MHHPA (Jansen Chimica, Geel, Belgium) was dissolved in dry acetonitrile and added to HSA in a 1:10 molar ratio (HSA:MHPA). The sample was adjusted to physiological pH, incubated overnight at 37°C, dialyzed (molecular weight (MW) cut-off 3500 Da; Spectrum, Gardena, CA) against PBS for 2 days, and then against 50 mM NH<sub>4</sub>HCO<sub>3</sub> for 3 days at 4°C. Dialysis buffer was freshly made each day. The MHHPA bound to the HSA was quantified by liquid chromatography-tandem mass spectrometry (LC/MS/MS) after hydrolysis (Kristiansson et al., 2003). The sample was evaporated to dryness and stored at -20°C. The internal standard (IS), HSA-[<sup>2</sup>H<sub>8</sub>]MHHPA conjugate, was prepared as described by Kristiansson et al. (2004).

### *Identification of covalent binding sites in HSA-MHHPA conjugates*

Using a modified methodology (Kristiansson et al., 2003), a nano-LC system (1100 series; Agilent Technologies, Waldbronn, Germany) coupled on-line with a nano-electrospray ionization (ESI) source on a hybrid quadrupole time-of-flight mass spectrometer (QqTOF; Q-STAR; Applied Biosystems, Foster City, CA) was used to analyze trypsinated HSA-MHHPA conjugate. Six picomoles of HSA-MHHPA conjugate dissolved in water was injected. The mass spectrometer was run in information-dependent acquisition (IDA) mode. Adducted peptides (Table 1) were identified and sequenced using Analyst 1.1 software.

### *Sample preparation*

Two milliliters of NAL from each sample were added to 300 pmol of IS. An aliquot of 100 µl dithiothreitol (DTT)

(1.2 mg/ml) was added to reduce disulfide bridges for 1 h at 55°C. Then, 100 µl iodoacetamide (1.4 mg/ml) was added and samples incubated for 30 min in darkness at room temperature. The samples were dialyzed, using a membrane with a MW cut-off at 3500 Da, in 1 L of 50 mM NH<sub>4</sub>HCO<sub>3</sub> at 4°C for 20 h. Five micrograms of trypsin (sequencing grade; Roche Diagnostics GmbH, Mannheim, Germany) was added to the samples which were then incubated at 37°C for 16 h. The samples were evaporated to dryness and stored at -20°C. Since no purified adducted tryptic HSA-MHHPA peptides were available, we decided to use a relative quantification approach in this pilot study. Thus, an in vitro-synthesized HSA-MHHPA conjugate was used for the calibrations. HSA-MHHPA standards were prepared to a final amount corresponding to 0, 10, 25, 35, 50, 75, 100, and 150 pmol hydrolyzed MHHPA. Each standard was prepared in 2 ml of pooled NAL (sample pooled from four different non-exposed persons), HSA-MHHPA conjugate, and IS.

### *LC/MS/MS of adducted peptides*

A LC system from Perkin Elmer (Series 200; Norwalk, CT) was used. The trypsinated HSA-MHHPA standard sample and the NAL sample were dissolved in 65 µl of water, and 15 µl was then injected into a C18 column (Jupiter 5u, 2.0 i.d. × 250 mm; Phenomenex, Torrance, CA). Water (A) and methanol (B), both acidified with 0.5% acetic acid, were used as mobile phase. The samples were separated using a gradient elution between 10 and 99% B for 20 min. The column was then reconditioned at 10% B for 10 min. The flow rate was 200 µl/min.

The column was connected to a MS/MS from Applied Biosystems (API 3000; Foster City, CA) with electrospray ionization (ESI). The mass spectrometer was operating in the positive ion mode. The sample was analyzed using selected reaction monitoring (SRM). Only two out of six adducted peptides had a response that was intense enough to be measured in the NAL: the peptides LK\*<sup>198</sup>CASLQK (Leu<sup>198</sup>-Lys<sup>205</sup> + MHHPA) and K\*<sup>525</sup>QTALVELVK (Lys<sup>525</sup>-Lys<sup>534</sup> + MHHPA), where \* denotes the adducted amino acid. The analytical parameters used are shown in Table 1.

**Table 1.** Transition values for trypsinated HSA adducted with MHHPA and the corresponding transition values for IS ([<sup>2</sup>H<sub>8</sub>]MHHPA adducted to HSA).

| Peptide                                        | Sequence                                        | z <sup>a</sup> | Transitions (m/z) | IS transitions (m/z) | CE <sup>b</sup> |
|------------------------------------------------|-------------------------------------------------|----------------|-------------------|----------------------|-----------------|
| Lys <sup>137</sup> -Arg <sup>144</sup> + MHHPA | K* <sup>137</sup> YLIEIAR                       | 2              | 612.5/528.4       | 616.5/528.4          | 29              |
|                                                |                                                 |                | 612.5/651.6       | 616.5/651.6          | 35              |
| Leu <sup>182</sup> -Lys <sup>195</sup> + MHHPA | LDEL <sup>182</sup> RDEGK* <sup>195</sup> ASSAK | 3              | 563.2/506.9       | 565.9/507.0          | 25              |
|                                                |                                                 |                | 563.2/229.3       |                      | 27              |
| Leu <sup>198</sup> -Lys <sup>205</sup> + MHHPA | LK* <sup>198</sup> CASLQK                       | 2              | 558.6/474.5       | 562.6/474.5          | 27              |
|                                                |                                                 |                | 558.6/706.5       |                      | 31              |
| Ala <sup>210</sup> -Arg <sup>218</sup> + MHHPA | AFK* <sup>210</sup> AWAVAR                      | 2              | 595.0/510.8       | 599.0/510.8          | 26              |
|                                                |                                                 |                | 595.0/673.5       | 599.0/673.5          | 28              |
| Leu <sup>349</sup> -Lys <sup>359</sup> + MHHPA | LAK* <sup>349</sup> TYETTTLEK                   | 2              | 733.2/649.2       | 737.2/649.2          | 32              |
|                                                |                                                 |                | 733.2/984.8       |                      | 35              |
| Lys <sup>525</sup> -Lys <sup>534</sup> + MHHPA | K* <sup>525</sup> QTALVELVK                     | 2              | 649.3/565.1       | 653.3/565.1          | 31              |
|                                                |                                                 |                | 649.3/771.6       |                      | 35              |

Note. The adducted amino acid is marked with an asterisk. <sup>a</sup>z denotes the charge of the peptide. <sup>b</sup>CE, collision energy.

There were two criteria which had to be fulfilled for an acceptable analysis. Samples which did not fulfill these criteria were denoted “not determined” (ND). The signal had to be three times higher than the noise, and the ratio between the two transitions for a peptide had to be the same as in the standard sample. The tryptic HSA-MHHPA standard conjugates formed a straight calibration curve with an *r*-value higher than 0.99.

Results

Symptoms, sensitization, and effect biomarkers

The symptoms and sensitization of the 12 workers, as well as levels of albumin, total protein, and ECP, are listed in Table 2. Five of the workers had symptoms from the upper airways and/or the eyes and three of them also had bleeding from the nose. One of the workers with upper airway symptoms also had symptoms from the lower airways. There seemed to be an association between sensitization, on the one hand, and albumin concentration and ECP level, on the other, at least before work.

Using Spearman’s rank correlation test, there was a correlation between albumin concentration and ECP level ( $R_s=0.75$ ,  $p=0.005$ ) before work on Monday but no correlation on Tuesday after work ( $R_s=0.18$ ,  $p=0.57$ ). The same was seen between total protein concentration and ECP level, with a correlation ( $R_s=0.87$ ,  $p<0.0001$ ) before work but not after work ( $R_s=0.28$ ,  $p=0.37$ ). There was a correlation between albumin and total protein concentration both before ( $R_s=0.85$ ,  $p<0.0001$ ) and after work ( $R_s=0.76$ ,  $p=0.005$ ). All subjects had levels below the detection limit for tryptase.

Identification of adducted peptides

The six peptides measured in the NAL were manually sequenced (Jeppsson et al., 2008) from product ion spectra obtained from IDA analysis using nano-LC-QqTOF to confirm the site of the MHHPA adduct. In these determinations, the trypsinated in vitro-synthesized HSA-MHHPA conjugate was used. The majority of the y-series ions were

observed in all spectra, identifying the sequence of the peptides; a smaller part of the b-ions were observed, also identifying the sequence. The adducted amino acid was observed on either y-ions or b-ions, pinpointing the adducted amino acid. An ion corresponding to the loss of MHHPA from the peptide sequence was observed in all product ion spectra. Figure 1 shows the product ion spectrum of Leu<sup>198</sup>-Lys<sup>205</sup> + MHHPA (LK\*<sup>\*</sup>CASQLK peak at *m/z* 558.6). The spectrum of the double-charged ion at *m/z* 558.6 showed a singly charged y<sup>+</sup>-series from y<sub>1</sub><sup>+</sup> to y<sub>6</sub><sup>+</sup>, identifying the peptide. The adducted b<sub>2</sub><sup>+</sup>-ion was observed at *m/z* 410.3. The double-charged ion at *m/z* 474.3 corresponds to the loss of MHHPA from the peptide.

Adducted peptides in NAL

Only two (peptide Leu<sup>198</sup>-Lys<sup>205</sup> + MHHPA and Lys<sup>525</sup>-Lys<sup>534</sup> + MHHPA) out of six adducted peptides described in Table 1 could be determined in the NAL samples. Chromatograms of the adducted peptide Leu<sup>198</sup>-Lys<sup>205</sup> + MHHPA before and after exposure for one of the subjects are shown in Figure 2.

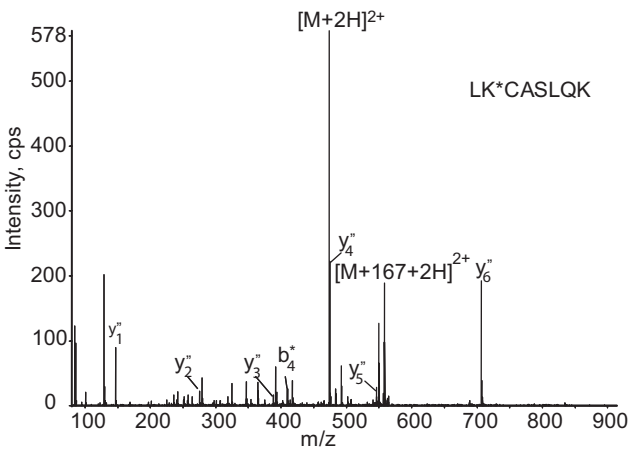


**Figure 1.** Product ion spectrum of the ion at *m/z* 558.6 corresponding to the adducted peptide LK\*<sup>\*</sup>CASQLK (Leu<sup>198</sup>-Lys<sup>205</sup> + MHHPA). The adduct is located on Lys<sup>199</sup>. The asterisk denotes the site of modification. The analysis was performed using nano-LC-QqTOF (liquid chromatography-quadrupole time-of-flight).

**Table 2.** Symptoms from eyes, nose, and lower airways during the 2 last days, sensitization and concentration of total protein (Prot.) albumin (Alb.), and eosinophil cationic protein (ECP) in the 12 subjects before work on Monday and after work on Tuesday.

| Subject | Symptoms         |                  |                           |  | Sensitization | Alb. before<br>(μg/ml) | Alb. after<br>(μg/ml) | Prot. before<br>(μg/ml) | Prot. after<br>(μg/ml) | ECP before<br>(μg/L) | ECP after<br>(μg/L) |
|---------|------------------|------------------|---------------------------|--|---------------|------------------------|-----------------------|-------------------------|------------------------|----------------------|---------------------|
|         | eyes and<br>nose | Nose<br>bleeding | Symptoms lower<br>airways |  |               |                        |                       |                         |                        |                      |                     |
| 1       | No               | No               | No                        |  | +             | 18                     | 24                    | 170                     | 81                     | 12                   | 2.1                 |
| 2       | No               | No               | No                        |  | +             | 20                     | 3.8                   | 100                     | 42                     | 26                   | 3.7                 |
| 3       | No               | No               | No                        |  | +             | 21                     | 0.8                   | 81                      | 47                     | 3.5                  | 3.8                 |
| 4       | No               | No               | No                        |  | –             | 6.4                    | 5.6                   | 54                      | 52                     | <2                   | <2                  |
| 5       | Yes              | Yes              | No                        |  | +             | 28                     | 2.4                   | 200                     | 36                     | 4.8                  | <2                  |
| 6       | Yes              | No               | No                        |  | –             | 5.0                    | 28                    | 72                      | 140                    | <2                   | <2                  |
| 7       | No               | No               | No                        |  | +             | 0.4                    | 0.7                   | 44                      | 46                     | <2                   | <2                  |
| 8       | Yes              | No               | Yes                       |  | –             | 2.6                    | 3.2                   | 37                      | 43                     | <2                   | <2                  |
| 9       | Yes              | Yes              | No                        |  | –             | 0.4                    | 0.2                   | 46                      | 12                     | <2                   | <2                  |
| 10      | Yes              | Yes              | No                        |  | +             | 37                     | 0.9                   | 150                     | 14                     | 2.8                  | <2                  |
| 11      | No               | No               | No                        |  | –             | 5.4                    | 3.0                   | 80                      | 46                     | 2.4                  | <2                  |
| 12      | No               | No               | No                        |  | –             | 5.5                    | 0.2                   | 53                      | 5                      | <2                   | <2                  |

The level of Leu<sup>198</sup>-Lys<sup>205</sup> + MHHPA was increased in eight subjects, and six of the subjects showed an increased level of Lys<sup>525</sup>-Lys<sup>534</sup> + MHHPA (Table 3). The response for Lys<sup>525</sup>-Lys<sup>534</sup> + MHHPA on the MS was lower than the response for Leu<sup>198</sup>-Lys<sup>205</sup> + MHHPA. A correlation between Leu<sup>198</sup>-Lys<sup>205</sup> + MHHPA and Lys<sup>525</sup>-Lys<sup>534</sup> + MHHPA after work ( $R_s=0.87$ ,  $p<0.0001$ ) was seen.

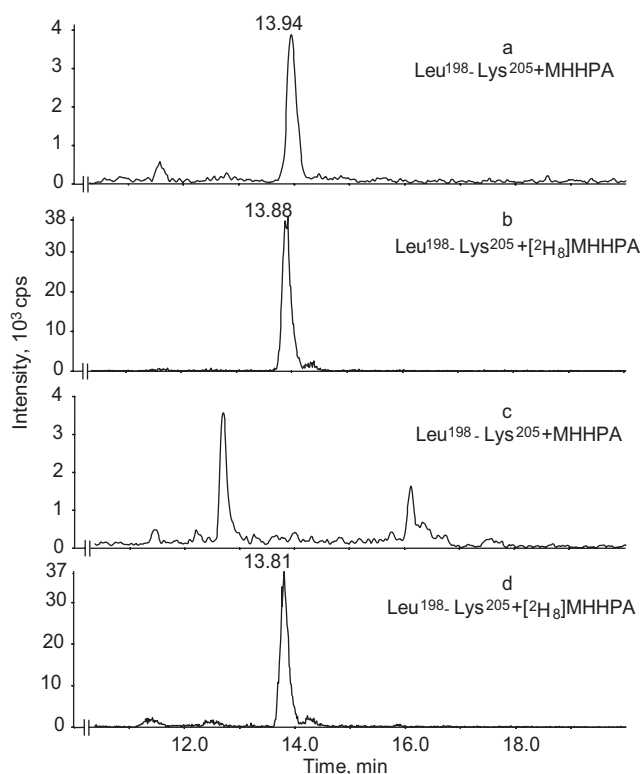


**Figure 2.** Extracted ion chromatogram of transitions  $m/z$  558.6/474.5 (a and c) and  $m/z$  562.6/706.5 (b and d) corresponding to the adducted peptide Leu<sup>198</sup>-Lys<sup>205</sup> + MHHPA and Leu<sup>198</sup>-Lys<sup>205</sup> + [<sup>2</sup>H<sub>8</sub>]MHHPA as internal standard. The extracted ion chromatogram in (a) is from subject 5 collected after 2 days of exposure, and the extracted ion chromatogram in (c) is from the same subject before exposure. (b and d) show extracted ion chromatograms for Leu<sup>198</sup>-Lys<sup>205</sup> + [<sup>2</sup>H<sub>8</sub>]MHHPA used in the after and before exposure samples from subject 5, respectively.

### Associations between urinary MHHP acid, adducts in NAL, and biological effects

There were no correlations between Leu<sup>198</sup>-Lys<sup>205</sup> + MHHPA or Lys<sup>525</sup>-Lys<sup>534</sup> + MHHPA and urinary levels of MHHP acid ( $R_s=0.018$ ,  $p=0.96$  and  $R_s=0.14$ ,  $p=0.66$ , respectively; Table 3). Also, there were no correlations between urinary MHHP acid and albumin or total protein levels ( $R_s=-0.24$ ,  $p=0.46$  and  $R_s=-0.18$ ,  $p=0.56$ , respectively).

No significant correlation was found between adducted peptides and albumin or total protein concentrations, but the correlations were rather high ( $R_s=0.33$ – $0.53$ ). In order to adjust for the different exposure levels, the peptides were adjusted for urinary levels of MHHP acid. After this adjustment the correlation increased to between 0.50 and 0.62. For the peptide Lys<sup>525</sup>-Lys<sup>534</sup> + MHHPA and albumin concentration after work, a significant correlation was obtained ( $R_s=0.62$ ,  $p=0.033$ ; Figure 3). There seemed not to be any associations between the levels of adducted peptides, on the one hand, and sensitization or symptoms during the last 2 days, on the other. There was no clear relationship between sensitization and symptoms.

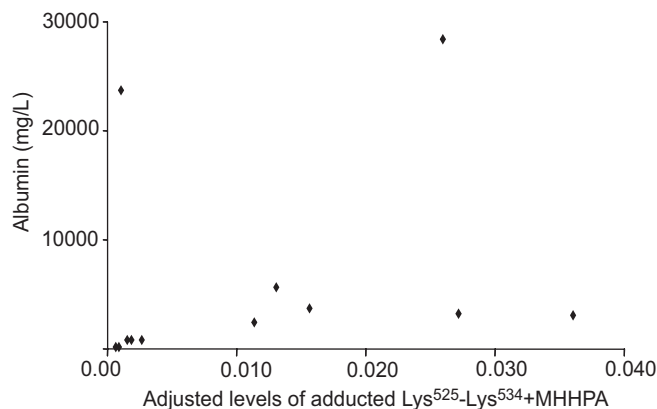


**Figure 3.** Plot of levels of the adducted peptide Lys<sup>525</sup>-Lys<sup>534</sup> + MHHPA, adjusted for urine concentration of MHHP acid, and the concentration of albumin in nasal lavages (NAL) after work. Spearman's rank correlation coefficient was 0.615 with a  $p$ -value of 0.033.

**Table 3.** MHHP acid in urine after work on Tuesday and measured levels of Leu<sup>198</sup>-Lys<sup>205</sup> + MHHPA and Lys<sup>525</sup>-Lys<sup>534</sup> + MHHPA in NAL before work on Monday and after work on Tuesday.

| Subject | Urine (nmol/mmol creatinine) | Leu <sup>198</sup> -Lys <sup>205</sup> + MHHPA before (pmol/ml NAL) | Leu <sup>198</sup> -Lys <sup>205</sup> + MHHPA after (pmol/ml NAL) | Lys <sup>525</sup> -Lys <sup>534</sup> + MHHPA before (pmol/ml NAL) | Lys <sup>525</sup> -Lys <sup>534</sup> + MHHPA after (pmol/ml NAL) |
|---------|------------------------------|---------------------------------------------------------------------|--------------------------------------------------------------------|---------------------------------------------------------------------|--------------------------------------------------------------------|
| 1       | 49                           | 0.4                                                                 | ND                                                                 | ND                                                                  | ND                                                                 |
| 2       | 19                           | 0.3                                                                 | 1.8                                                                | ND                                                                  | 0.3                                                                |
| 3       | 27                           | 0.5                                                                 | 3.6                                                                | ND                                                                  | ND                                                                 |
| 4       | 108                          | 0.4                                                                 | 4.5                                                                | ND                                                                  | 1.4                                                                |
| 5       | 224                          | ND                                                                  | 10.4                                                               | ND                                                                  | 2.5                                                                |
| 6       | 46                           | 0.6                                                                 | 10.8                                                               | 0.1                                                                 | 1.2                                                                |
| 7       | 58                           | 0.3                                                                 | 0.7                                                                | 0.3                                                                 | 0.2                                                                |
| 8       | 12                           | 0.4                                                                 | 1.0                                                                | ND                                                                  | 0.3                                                                |
| 9       | 82                           | 0.4                                                                 | ND                                                                 | ND                                                                  | ND                                                                 |
| 10      | 33                           | 0.9                                                                 | ND                                                                 | ND                                                                  | ND                                                                 |
| 11      | 19                           | 0.4                                                                 | 3.8                                                                | 0.6                                                                 | 0.7                                                                |
| 12      | 59                           | ND                                                                  | ND                                                                 | ND                                                                  | ND                                                                 |

Note. ND, not determined.

## Discussion

The main result of this study is that protein adducts can be analyzed in vivo after low occupational exposures to MHHPA. The results also show that there is a correlation between exposure-adjusted concentrations of adducted peptides and albumin levels in NAL.

Only 12 workers were included in this study. This number is sufficient to investigate whether MHHPA adducted HSA peptides can be found in NAL. However, this is too few to investigate associations between the level of MHHPA adducted HSA peptides, on the one hand, and health effects and data on biomarkers of effect in exposed workers, on the other. Thus, this study must be regarded as a pilot study for such associations. The reason for the few subjects is that studies of this kind are very time-consuming. It is often therefore best to start with a pilot study to see if it is worth performing a larger study. Unfortunately, the plant was moved to another country during the analyses. Thus, we have no possibility to continue the investigation at the same plant, and we have no information on other plants in Sweden where MHHPA is used. However, the method may be important in studies of other OAAs, such as phthalic anhydride, a widely used OAA.

It is often not meaningful to use statistics to a large extent with only 12 subjects. Therefore, most data were analyzed in a subjective way only. However, we decided to still use a non-parametric test, Spearman's rank correlation test, to look at correlations between continuous variables, but, of course, the size of the study group decreased the possibilities to obtain significant results.

The fraction of workers with airway diseases was in accordance with other larger studies on workers exposed to MHHPA (Nielsen et al., 2001, 2006; Rosqvist et al., 2003). In addition, the pattern of more workers with symptoms from the eyes and upper airways but fewer with symptoms from the lower airways is consistent with previous studies. Also, several of the workers were sensitized against MHHPA. This is similar to previous studies (Nielsen et al., 2001; Rosqvist et al., 2003; Welinder et al., 1994, 2001). There was no clear association between symptoms and sensitization, which is in accordance with the fact that about half of symptomatic workers do not have OAA-specific IgE antibodies (Nielsen et al., 1992; Rosqvist et al. 2003).

Albumin in NAL has previously been used as a biomarker of effect in workers exposed to OAAs. Nielsen et al. (1994) exposed sensitized workers with symptoms, non-sensitized workers with symptoms, and non-sensitized workers without symptoms by flushing the nostrils with a solution containing a conjugate between HSA and HHPA. Albumin and some other mediators were then analyzed in NAL. There was a clearly significant increase in albumin and symptoms in the sensitized group, but not in the two groups of non-sensitized workers. This shows that the conjugate with HHPA is biologically active, which was also supported by the study of Zhang et al. (1998), who showed that it is possible to sensitize animals via conjugates between OAAs and HSA. Furthermore,

the study by Nielsen et al. (1994) indicates that albumin is a good biomarker of effect in OAA-sensitized workers.

Total protein concentration is a less used biomarker of effect. However, we found a high correlation between albumin and total protein levels. This is not surprising, since much of the protein in NAL is albumin. In addition, since the origin of albumin in NAL probably is through exudation of plasma, several other proteins from the plasma will be present in the NAL.

There was no correlation between urinary levels of MHHP acid and MHHPA adducts. This might indicate that there is a large difference between individuals in the formation of adducts for MHHPA in NAL. Such a difference has previously been reported for HHPA-HSA adducts (Kristiansson et al., 2004).

We found adducted peptides in the NAL on Monday morning following a weekend without exposure; some levels were in the same range as after the exposure on Tuesday afternoon. Thus, the half-life of the conjugates between MHHPA and albumin in the nose is longer than what might be expected. This has also been described in subjects exposed to HHPA, where adducted peptides could be detected 3 days after the exposure (Johannesson et al., 2004). Thus, the adducted HSA in the nose can be regarded as a biomarker of exposure over a rather long period of time. There is no possibility that the origin of the adducts is from plasma exudation of adducted albumin, since the levels in plasma are so extremely low (Rosqvist et al., 2001).

It is interesting to note that ECP, a marker of inflammation, was significantly associated with albumin before the work-shift on Monday but not after the work-shift on Tuesday. The same was seen for total protein concentration and ECP. Moreover, there seemed to be a relation between sensitization and ECP and albumin before the shift. It would be expected that the associations were stronger after the exposure on Tuesday, but this association was less clear after the work-shift. One reason for this observation might be that the turnover of proteins in the nose is rather slow. It is probable that the conjugates between MHHPA and albumin are biologically active, just like the HHPA conjugates (Nielsen et al., 1994), which could give rise to the associations between sensitization and effect biomarkers found on Monday morning. It is also possible that there is a variation in the protein content during the day. The low levels of tryptase indicate little activation of mast cells despite the presence of MHHPA-specific IgE antibodies in several subjects.

The levels of MHHPA in air can be estimated from the level of MHHP acid in the urine (Lindh et al., 1997). From the urinary MHHP acid levels in this study, it was calculated that the average air level in the exposed workers was  $9 \mu\text{g}/\text{m}^3$  (range  $2\text{--}32 \mu\text{g}/\text{m}^3$ ). Despite this low exposure level, it was possible to analyze adducted peptides in most of the samples. Still, the analytical method is rather easy to apply.

It seems probable that the concentration of adducts in the nose is related to the risk of sensitization and health effects. In the study by Kristiansson et al. (2004), there

seemed to be an individual difference in concentrations of adducts. We therefore chose to use the actual concentration of the adducts rather than the albumin-adjusted adduct levels.

In the present study there were rather high correlations between the levels of adducted peptides and albumin. However, this might be expected; it is easy to draw the conclusion that the more albumin there is in the nose, the more adducts are formed. In the study on HHPA by Kristiansson et al. (2004) there was indeed a correlation between the peptide adducts and albumin in the nose (MH Kristiansson, personal communication), which further supports this. Moreover, in both studies, the correlations were increased when the adduct levels were adjusted for the exposure. This probably indicates that formation is dependent also on exposure levels, which might be expected, although we did not find such an association between urinary MHHPA acid and peptide adducts. In the study by Kristiansson et al. (2004) such a relationship was found.

Although it might be expected that most of the correlation between peptide adduct levels and albumin has its origin in the albumin content in the nose and the exposure, it cannot be ruled out that the exposure to MHHPA in part may induce an exudation of plasma into the nose. The fact that two individuals have deviating levels of albumin in Figure 3 indicates that there are more factors determining adduct levels than the albumin levels and the exposure. Furthermore, there is a possibility that some individuals with increased levels of albumin in the NAL are more susceptible to develop hypersensitivity against low molecular weight compounds because of the higher levels of adducts generated.

The ability to form adducts seems to be different for different amino acids. The adduction of Lys<sup>199</sup> has been described in other studies (Ding et al., 1993; Kristiansson et al., 2003; Noort et al., 2000; Olsen et al., 2003; Walker, 1976; Yvon & Wal, 1988) as one of the most reactive sites in HSA. The peptide Leu<sup>198</sup>–Lys<sup>205</sup>, containing Lys<sup>199</sup>, is the adducted peptide with the highest response in our study. However, the fact that Lys<sup>525</sup>–Lys<sup>534</sup> + MHHPA with the adduct on Lys<sup>525</sup> shows a higher correlation with albumin concentration than Leu<sup>198</sup>–Lys<sup>205</sup> + MHHPA may suggest that there is a difference in the potential to induce hyperresponsiveness between these peptides.

No correlation could be found between the health effects and the level of MHHPA adducts. This may be due to the small group of subjects; in general, a substantially larger group of subjects is required to find such relationships.

## Conclusions

In conclusion, in this study, MHHPA adducts in NAL from low-exposed workers could be analyzed. The adducted peptides were found to correlate with some biomarkers of effect. Most of this correlation probably has its origin in

that individuals with high albumin levels in the nose also form more albumin adducts, but might also be a result of exudation of albumin from the plasma. Furthermore, it is possible that the HSA adduct binding site is important for the effect. Further investigations in larger study groups have to be performed before any substantial conclusions can be made.

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