Scand J Work Environ Health 2001;27(2):133-139

<https://doi.org/10.5271/sjweh.600>

Issue date: Apr 2001

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Refers to the following texts of the Journal: [1997;23\(3\):214-220](#)
[1998;24\(3\):220-227](#)

The following article refers to this text: [2003;29\(4\):297-303](#)

Key terms: [allergenic hexahydrophthalic](#); [biological monitoring](#); [biomarker](#); [blood](#); [gas chromatography](#); [long-term exposure](#); [mass spectrometry](#); [methylhexahydrophthalic anhydride](#); [occupational exposure](#); [organic acid anhydride](#); [total plasma protein adduct](#); [toxicokinetics](#); [type-1-allergy](#); [urine](#)

This article in PubMed: www.ncbi.nlm.nih.gov/pubmed/11409596

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Total plasma protein adducts of allergenic hexahydrophthalic and methylhexahydrophthalic anhydrides as biomarkers of long-term exposure

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Rosqvist S, Johannesson G, Lindh CH, Jönsson BAG. Total plasma protein adducts of allergenic hexahydrophthalic and methylhexahydrophthalic anhydrides as biomarkers of long-term exposure. *Scand J Work Environ Health* 2001;27(2):133–139.

Objectives The aim of this study was to evaluate the applicability of total plasma protein adducts (TPPA) of 2 sensitizing low-molecular-weight allergens, hexahydrophthalic anhydride (HHPA) and methylhexahydrophthalic anhydride (MHHPA), as biomarkers of long-term exposure.

Methods Urine samples from occupationally exposed workers were analyzed for the levels of urinary metabolites of HHPA and MHHPA, and the levels were used as the index of exposure. In addition, blood samples were obtained from the same persons, and the levels of TPPA were determined. Reversed solid phase extraction, derivatization using pentafluorobenzyl bromide, and gas chromatography-mass spectrometry analysis in the negative ion chemical ionization mode were used to quantify the exposure. To assess the suitability of TPPA as a biomarker of exposure to the anhydrides, the TPPA levels were correlated to urinary metabolite levels and hemoglobin (Hb) adducts. The toxicokinetics of TPPA were also studied to determine the elimination half-time of the adducts.

Results The levels of TPPA correlated exceptionally well with the metabolite levels in the urine sampled repeatedly, giving $r=0.97$ for HHPA and $r=0.92$ for MHHPA. The TPPA of HHPA correlated highly with the Hb adducts with $r=0.86$. There were also good correlations between single urinary determinations and the TPPA levels ($r_s=0.71$ and 0.81 , respectively, for HHPA and MHHPA). The in vivo decay of TPPA gave an elimination half-time of 22 days for HHPA and 24 days for MHHPA.

Conclusions TPPA levels of HHPA and MHHPA are excellent biomarkers of long-term exposure to anhydrides.

Key terms biological monitoring, blood, gas chromatography, mass spectrometry, occupational exposure, organic acid anhydrides, toxicokinetics, type-I-allergy, urine.

Organic acid anhydrides (OAA) are reactive, low-molecular-weight compounds used extensively as cross-linking agents and starting raw materials in the chemical industry. They are also known to be extremely powerful occupational allergens. Symptoms of diseases such as asthma, rhinitis, and conjunctivitis have been found in up to two-thirds of exposed workers (1–3). Specific immunoglobulin (Ig) E against conjugates between an anhydride and human serum albumin (HSA) have been found to be present in more than 60% of exposed workers (4). Hexahydrophthalic anhydride (HHPA) and methylhexahydrophthalic anhydride (MHHPA) are 2

anhydrides that are especially sensitizing; they have been shown to elicit immunologic responses in exposed workers at levels even below $10 \mu\text{g}/\text{m}^3$ (5, 6). Thus there is a need for reliable, practical, and sensitive methods for monitoring exposure for use in health surveillance at the workplace.

There are methods available for the biological monitoring of exposure to these anhydrides; the methods measure the amounts of free corresponding acids; hexahydrophthalic acid (HHP acid) and methylhexahydrophthalic acid (MHHP acid), respectively, in urine and plasma (7–12). However, the half-time of HHP and

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MHHP acids in urine is only about 5–6 hours, and thus a single urine sample shows only 1 day's exposure (9, 12). Long-term exposure can be assessed through repetitive sampling, but such sampling tends to be tedious and impractical, demanding much time and discipline. Biomonitoring using adducts between endogenous proteins and xenobiotics as biomarkers of exposure was an idea first suggested by Ehrenberg and his co-workers (13). These adducts are superior biomarkers in the sense that just 1 sample can give information about long-term exposure. Reports on hemoglobin (Hb) adducts of HHPA and MHHPA have been published earlier (14–16). These adducts have the advantage of monitoring cumulative exposure during the lifetime of the protein (4 months), but the degree of adduction tends to be low, especially for electrophilic xenobiotics, due to the erythrocyte membrane hindering access to Hb (17, 18). This phenomenon may result in poor detection limits when low exposures cannot be accurately and reliably quantified. In contrast, HSA may constitute a better index of integrated cumulative exposure, as it is easily accessible by the xenobiotics and is also abundant. Several methods have been published on the quantification of HSA adducts of various compounds (19–21). The common drawback in all these procedures is the laborious and tedious protein purification steps that make the methods impractical for bulk routine use.

In this study we took the simpler and easier approach of using the total plasma protein adducts (TPPA) of HHPA and MHHPA as the index of the exposure. The use of these adducts for the biomonitoring of long-term exposure to the anhydrides has been evaluated.

Subjects and methods

Subjects

The workers investigated were employed at a plant manufacturing electrical capacitors. They were exposed to HHPA and MHHPA mainly through the inhalation of the vapors. All of the workers had been exposed for at least 4 months prior to the investigation. Permission for the study was obtained from the Ethics Committee at the Lund University, and all the participating workers gave an informed consent.

Collection of biological samples

In an earlier study, long-term exposure to HHPA and MHHPA was assessed through the analysis of urine samples collected repeatedly from 10 exposed workers, during the last 4 hours of the workshift, on 10 to 12 different days, over a period of 4 weeks (14). The mean

urinary HHP acid levels over the 4-week period were found to be in the range 76–3300 nmol/mmol creatinine. The mean urinary levels of MHHP acid ranged from 0 to 56 nmol/mmol creatinine. Blood was also sampled from the same 10 workers at the end of the 4-week period and was used to determine the Hb adduct levels. In the previous work (14), the mean HHPA-Hb adduct levels ranged from 0.45 to 25 pmol/g Hb. In our study, corresponding plasma from the same 10 workers was analyzed for the TPPA levels. In addition, urine samples from a total of 117 HHPA- and 116 MHHPA-exposed workers were collected during the last 4 hours of an 8-hour workshift. Blood samples for the determination of TPPA were also collected the same week from all the workers from which urine samples were obtained.

For the study of the kinetics of TPPA, blood samples were collected from 1 HHPA-exposed worker and 1 MHHPA-exposed worker who left employment and were thereafter unexposed. Blood samples from these 2 workers were taken on their last day at work and then once a month over 4 months after the termination of employment. We also collected blood samples from 5 HHPA-exposed and 3 MHHPA-exposed workers before and after a 28- to 35-day summer vacation to see how well the monitored TPPA levels compared with theoretically predicted adduct levels.

All the blood samples were drawn from an antecubital vein and collected in 10-ml Venoject® blood sampling tubes (Terumo Europe, Leuven, Belgium) containing sodium heparin. The blood was allowed to cool to room temperature, it was then centrifuged at 1500 g for 10 minutes, and the plasma was then separated. The urine and plasma samples were stored in polyethylene test tubes at –20°C until the analysis.

Determination of hexahydrophthalic acid and methylhexahydrophthalic acid in urine

The levels of HHP acid and MHHP acid in urine were determined as described elsewhere (10). Briefly, the acids were worked-up using reversed, solid-phase extraction (SPE) and then derivatized using pentafluorobenzyl bromide (PFBBBr) and analyzed by gas chromatography-mass spectrometry (GC-MS) in the negative-ion chemical ionization (NICI) mode.

Determination of total plasma protein adducts of hexahydrophthalic anhydride and methylhexahydrophthalic anhydride

The levels of HHPA and MHHPA bound to plasma proteins were determined as published recently (22). Thus aliquots of plasma were dialyzed, and the bound HHPA and MHHPA were hydrolyzed from the proteins to the corresponding acids. The acids were then purified by

SPE, derivatized to the corresponding esters using PFBBR and analyzed by GC-MS in the NICI mode.

Statistical analysis

Spearman's rank correlation coefficient (r_s) was used to analyze the results in cases when the data were distributed mainly in clusters with some outliers. Simple linear regression was used to describe the correlations between the pairs of values when the data were evenly spread between the lowest and the highest values.

Results

Correlations between total plasma protein adducts and urinary metabolite levels

The levels of TPPA of HHPA in the plasma of the 10 persons for whom frequent urine sampling was performed were between 700 and 13 400 fmol/ml. The TPPA levels of HHPA and the mean urinary HHP acid levels were very strongly correlated with a linear regression of 0.97 ($P=0.0001$, figure 1). The levels of the protein adducts of MHHPA in the plasma of the 10 workers ranged between 0 and 830 fmol/ml. Again a very strong linear correlation with $r = 0.92$ ($P=0.0001$) was found between the MHHPA TPPA levels and the mean urinary MHHP acid levels (figure 2). Furthermore, when the gradients of the 2 plots were compared, it could be seen that, at the same exposure levels, the amounts of MHHPA adducts were about 3 times higher than those of HHPA.

Figure 1. Correlation between the mean levels of hexahydrophthalic (HHP) acid in urine collected on 10–12 occasions over a period of 4 weeks from 10 workers exposed to hexahydrophthalic anhydride (HHPA) and the levels of the total plasma protein adducts (TPPA) of HHPA at the end of the period. One unexposed subject was included at zero exposure. The correlation coefficient was 0.97.

The range of the HHPA TPPA levels in the plasma of the 117 workers was 0–10 700 (mean 800, median 92) fmol/ml, and the range of the HHP acid levels in urine was 6–3060 (mean 170, median 21) nmol/mmol creatinine. The plot of the HHPA TPPA against the urinary HHP acid levels is shown in figure 3. In Spearman's rank correlation test, the HHPA TPPA levels significantly correlated with the urinary HHP acid levels ($r_s=0.71$, $P=0.0001$).

The range of the MHHPA TPPA levels in the plasma of the 116 workers was 0–40 300 (mean 1860, me-

Figure 2. Correlation between the mean levels of methylhexahydrophthalic (MHHP) acid in urine collected on 10–12 occasions over a period of 4 weeks from 10 workers exposed to methylhexahydrophthalic anhydride and the levels of the total plasma protein adducts (TPPA) of MHHPA at the end of the period. One unexposed subject was included at zero exposure. The correlation coefficient was 0.92.

Figure 3. Correlation between the urinary levels of hexahydrophthalic (HHP) acid and hexahydrophthalic anhydride (HHPA) plasma protein adducts from 117 exposed workers. The urinary levels of HHP acid were determined in urine sampled during the last 4 hours of 1 workshift, and they represent the exposure mainly during that day. Plasma protein adducts of HHPA were analyzed in a single blood sample, and they represent long-term exposure. The Spearman's rank correlation coefficient was 0.71.

dian 370) fmol/ml, and the range of the levels of MHHP acid in urine was 6–1730 (mean 130, median 33) nmol/mmol creatinine. Figure 4 shows the plot of MHHPA TPPA against the urinary MHHP acid levels. The TPPA levels of MHHPA and the urinary MHHP acid levels assessing the short-term exposure also correlated significantly with $r_s=0.81$ ($P=0.0001$).

Correlations between total plasma protein adducts and hemoglobin adducts of hexahydrophthalic anhydride

The HHPA TPPA levels of the 10 workers were found to correlate highly with the Hb adduct levels, as deter-

Figure 4. Correlation between the urinary levels of methylhexahydrophthalic (MHHP) acid and plasma-MHHPA adducts from 116 exposed workers. The urinary levels of MHHP acid were determined in urine sampled during the last 4 hours of 1 workshift, and they represent the exposure mainly during that day. Plasma adducts of methylhexahydrophthalic anhydride (MHHPA) were analyzed in a single blood sample, and they represent long-term exposure. The Spearman's rank correlation coefficient was 0.81.

Figure 5. Correlation between the levels of total plasma protein adducts (TPPA) and hemoglobin (Hb) adducts of hexahydrophthalic anhydride (HHPA). One unexposed subject was included at zero exposure. The correlation coefficient was 0.86.

mined in a previous study (14), with $r=0.86$ ($P=0.0006$). The relationship is shown in figure 5.

In vivo stability of total plasma protein adducts

The decline of the adducts after the end of the occupational exposure is shown in figure 6. With the assumption of first order elimination kinetics, the half-times of the adducts were calculated using the linear regression of a plot of the natural logarithm of the adduct versus the postexposure time. The calculated half-time of the HHPA TPPA was 22 days, and that of MHHPA was 24 days. TPPA levels in plasma collected from 5 HHPA-exposed workers and 3 MHHPA-exposed workers before and after a 28- to 35-day vacation were quantified to see how the monitored values compared with

Figure 6. Levels of total plasma protein adducts (TPPA) in plasma from a worker exposed to hexahydrophthalic anhydride (HHPA) (closed circles) and from a worker exposed to methylhexahydrophthalic anhydride (MHHPA) (triangles). The samples were collected once a month over a period of 4 months after the termination of employment.

Table 1. Monitored and predicted decay of total plasma protein adducts (TPPA) of hexahydrophthalic anhydride (HHPA) and methylhexahydrophthalic anhydride (MHHPA) in exposed workers before and after a 28- to 33-day vacation.

Anhydride	Before vacation	After vacation	
	Monitored amount of TPPA (pmol/ml plasma)	Monitored amount of TPPA (pmol/ml plasma)	Predicted amount of TPPA ^a (pmol/ml plasma)
HHPA	5.00	1.62	1.60
HHPA	4.18	1.24	1.59
HHPA	0.60	0.20	0.18
HHPA	0.48	0.15	0.15
HHPA	0.35	0.20	0.13
MHHPA	0.15	0.04	0.05
MHHPA	0.14	0.06	0.05
MHHPA	0.13	0.12	0.04

^a Calculated theoretically, using a half-time of 20 days.

theoretically predicted values on the assumption of a half-time of 20 days in plasma. The results are summarized in table 1.

Discussion

Our major finding was that the TPPA levels of the 2 potent allergenic, low-molecular-weight chemicals HHPA and MHHPA are excellent indices of long-term exposure to these anhydrides.

Although the concept of protein adducts as a dosimeter of exposure has been around since 1974, progress with this type of monitoring has been slow, with remarkably few publications on the subject, and their use in exposure monitoring is still very limited, probably due to the need for special and advanced analytical instrumentation coupled with laborious and tedious sample work-up procedures. But now, as mass spectrometers are becoming more readily and cheaply available, there are better opportunities to investigate the full potential of this approach.

Some efforts have also been made to develop simpler work-up procedures (23). In this report we have used a recently developed method to quantify the levels of TPPA (22). This method is easy and convenient, and it dispenses with tedious, labor-intensive protein isolation steps. The protocol consists of simple steps such as dialysis, quick and mild hydrolysis, and one-step derivatization. It is also practical, using a minimum of basic reagents found commonly in most laboratories. However, it should be pointed out that this method is limited to lipophilic and hydrolyzable adducts.

The correlations between the levels of TPPA and long-term exposure as determined by repetitive urine sampling were exceptionally good for both HHPA and MHHPA. The correlation between the presented TPPA method and short-term biological monitoring using 1-day urine sampling was slightly lower, and this result was to be expected. Protein adducts reflect mean integrated, long-term exposure over a period of several weeks, and they are relatively less dependent on sampling time, whereas urinary metabolites reflect only the current (1-day) exposure from 1 workshift, which is influenced by variations in exposure patterns during the day with alternating peak, low, or periodic exposures.

There may be reasons to expect some problems with the precision of the biological monitoring method using TPPA, as the levels of protein between and within persons tend to vary. Therefore, if the level of exposure is quantified in terms of milliliters of plasma, a negative effect on the precision can be expected. However, we did not find any such effect in this study since we saw excellent correlations between exposure and the

TPPA levels. In fact, the obtained correlations were far better than any previously reported associations between exposure and adduct levels in general. In addition, as reported in an earlier study, we found high correlations between the TPPA levels and the adducts with merely the HSA in 10 workers with differing protein levels (22). Furthermore, in this study, we found a close correlation also between TPPA and the Hb adducts of HHPA.

Adducts are conveniently stable, with a half-time that corresponds well with the half-time of HSA, and this phenomenon suggests that albumin may be the major adducting protein in plasma. This assumption agrees with the results of other studies (24). This is an interesting finding since conjugates between HSA and anhydrides have been used for many years in tests for specific antibodies. In addition, *in vitro* synthesized conjugates between serum albumin and anhydrides have been shown to induce the production of specific antibodies in rats (25).

In this study we found that a mean urinary MHHP acid level of 50 nmol/mmol creatinine corresponds to a TPPA level of about 650 fmol/ml. In a previous study in the same factory we found that a urinary MHHP acid level of 50 nmol/mmol creatinine corresponded to about 7 $\mu\text{g}/\text{m}^3$ (12). The detection limit of the TPPA method for MHHPA is about 30 fmol/ml. Thus detectable levels of adduct are formed at average air levels corresponding to less than 1 $\mu\text{g}/\text{m}^3$. There is a definite need for monitoring such low exposure levels because it has been reported that up to 20% of workers exposed to HHPA and MHHPA below 10 $\mu\text{g}/\text{m}^3$ have anhydride-specific IgE antibodies (5). The lowest exposure levels that can be monitored by the Hb adduct method previously described is an order of magnitude higher. This TPPA method is also capable of monitoring high exposures; levels up to more than 500 $\mu\text{g}/\text{m}^3$ can be quantified. Thus HHPA and MHHPA TPPA are suitable and appropriate dosimeters for variable exposure assessments.

In the monitoring of exposure to carcinogenic compounds, protein adducts are often considered to be biomarkers of effective dose (ie, they indicate the dose of the compound at the target site) (20). The formation of protein adducts from allergenic, low-molecular-weight chemicals is believed to be an important step in the IgE sensitization process. Thus there are reasons for investigating the possibility that these protein adducts may also be usable as biomarkers of effective dose and exposure. In this study we found that the 2 anhydrides behave differently, MHHPA giving more adducts than HHPA for the same levels of exposure. It would therefore be interesting to see whether MHHPA is indeed somewhat more sensitizing than HHPA. Welinder et al (26) studied the sensitizing potentials of 13 cyclic acid anhydrides following the intradermal injection of the

anhydrides into guinea pigs and found a wide range of responses. However, the responses to HHPA and MHHPA in their study seemed to be almost equal. Similar results were obtained by Zhang and his co-workers (25) when they studied antibody levels from 14 anhydrides after intradermal injection into rats. Currently no studies of occupationally exposed workers are available indicating whether MHHPA is more sensitizing than HHPA. This possibility should be studied further.

We have, in this work, shown that the TPPA levels of HHPA and MHHPA are excellent biomarkers for assessing long-term exposures to these chemicals. Thus we have used low-molecular-weight allergens to assess the applicability of TPPA as an index of exposure, but this approach can also be applied in studies of the exposure-response and dose-response relationships of other xenobiotics.

Acknowledgments

The excellent technical assistance given by Ms K Olson, Ms H Ottosson and Mr KO Lennartsson is gratefully acknowledged.

This work was supported by grants from the Swedish Medical Research Council (11336), the Swedish Council for Work Life Research, the Vårdal Foundation, the European Union (BMH4-CT-98-3951), and the Medical Faculty at Lund University.

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Received for publication: 18 September 2000