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Exposure-response relationships for hexahydrophthalic and methylhexahydrophthalic anhydrides with total plasma protein adducts as biomarkers

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Objectives This study investigated the exposure-response relationships of hexahydrophthalic anhydride (HHPA) and methylhexahydrophthalic anhydride (MHHPA) and evaluated the applicability of the total plasma protein adducts (TPPA) of these anhydrides as biomarkers of exposure and risk.

Methods In a cross-sectional study of 139 workers in a plant manufacturing electrical capacitors, the long-term exposure to HHPA and MHHPA was assessed through the quantification of TPPA using gas chromatography–mass spectrometry. Smoking and medical histories were obtained through questionnaires. Work-related symptoms of the eyes and airways were recorded. Specific immunoglobulin (Ig) E (radioallergosorbent test) and IgG (enzyme-linked immunosorbent assay) were determined in serum.

Results The mean level of the TPPA of HHPA was 840 fmol/ml and that of the TPPA of MHHPA was 1700 fmol/ml. There was no correlation between the TPPA of HHPA and the TPPA of MHHPA. Of all the workers, 19% were found to be positive for specific IgE and 17–19% for IgG. Positive associations were observed between HHPA exposure and specific IgE and IgG and between MHHPA exposure and specific IgG. Regarding work-related symptoms, 27% of the workers had symptoms of the nose, 21% had symptoms of the eyes, 11% had symptoms of the lower airways, and 8% had nose bleeding. There were significant exposure-response relationships for symptoms of the eyes and nose for HHPA exposure.

Conclusions The results show that there is an exposure-response relationship for HHPA both with specific antibodies and with work-related symptoms and down to adduct levels of 40 fmol/ml plasma. In addition, the results elucidate the potential power of TPPA as a relevant index of exposure and risk.

Key terms exposure-response relationships, phthalic anhydrides, protein adducts.

Organic acid anhydrides (OAA) are a family of industrial chemicals used extensively in the manufacturing of a range of everyday products such as plastics, textiles, paints, and electronic components. They are also known to be powerful inducers of airway diseases. Symptoms such as asthma, rhinitis, and conjuctivitis have, in some studies, 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) were present in more than 60% of exposed workers (4). Hexahydrophthalic anhydride (HHPA) and methylhexahydrophthalic anhydride (MHHPA) are two particularly potent OAA (5–7). However, currently, no occupational exposure limits exist for these two substances in Europe or elsewhere. Thus there is a strong need for studies on the exposure-response relationships of HHPA and MHHPA. Such studies require good methods for accurate exposure assessments that can be used in investigations of groups consisting of rather large numbers of people.

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The concept of protein adducts as indices of long-term exposure to xenobiotics was first suggested by Ehrenberg and his co-workers in 1974 (8). However, despite the potential of protein adducts as biomarkers of exposure, development in the area has been remarkably slow, probably because of the rather laborious laboratory procedures coupled with the high costs of the equipment needed for analyzing the samples. Recently, we reported a convenient method for analyzing the total plasma protein adducts of HHPA and MHHPA (9) and showed that these adducts are excellent biomarkers of long-term exposure to the anhydrides (10).

The aim of our present study was to investigate the exposure-response relationships for workers occupationally exposed to HHPA and MHHPA. The exposures were quantified using the total plasma protein adducts of HHPA and MHHPA, and the responses were assessed using levels of specific immunoglobulins (Ig) and work-related symptoms.

Subjects and methods

The plant

The plant produced electrical capacitors where the components were fixed and isolated using an epoxy resin with HHPA and MHHPA as hardeners. Production started 32 years before our present study. Determinations of HHPA in air were started 5 years before the investigation, and the air levels at the time of the study were approximately 50% of those 5 years earlier. Determinations of MHHPA in air were started 3 years before the investigation, and the air levels at the time of the study were approximately five times higher than at the start of the monitoring. There were different departments in the plant, some using 100% HHPA, others using 100% MHHPA, and some using both HHPA and MHHPA in different proportions. This variation explains the lack of correlation between the adducts of HHPA and MHHPA. (See the Results section.) Other chemicals used were epichlorohydrin-based epoxy resin, dimethylbenzylamine (DMBA), and acetone. No other OAA was ever used in the plant. The workers were exposed mainly to the vapor of the anhydrides, from leaking curing ovens, casting, and hot components that were transferred directly from the ovens to other departments.

Subjects

Altogether 157 workers were invited to take part in the study, of which 154 participated. The relationships between the exposure, assessed using air sampling and urinary metabolite levels, and specific antibodies and work-related symptoms have previously been reported for this group (7). Plasma for the adduct analysis of our present study was obtained from 139 of the 154 workers. The median employment time was 4 (range 0–29) years. As referents, 57 subjects from two mechanical industries were employed, but unfortunately plasma samples were not obtained from the workers. Permission for this study was obtained from the Ethics Committee of the Lund University.

Exposure assessments

Plasma was obtained from the workers and analyzed for the total plasma protein adducts of the anhydrides, the adduct levels then being used as exposure indices. The total plasma protein adducts of HHPA and MHHPA were quantified using a recently published gas chromatographic–mass spectrometric (GC–MS) method (9). Briefly, aliquots of plasma were dialyzed, and the adducted HHPA and MHHPA were hydrolyzed from the proteins to the corresponding acids. The acids were purified using solid phase extraction, derivatized using pentafluorobenzyl bromide, and analyzed by GC–MS in the negative ion chemical ionization mode.

The reference limits of the occupational exposure were determined to be 40 and 20 fmol/ml for HHPA and MHHPA, respectively. These limits were defined as the mean of the concentration of the unexposed subjects plus two times the standard deviation of these concentrations. The concentrations determined for the unexposed workers were due to contamination of HHP and MHHP acid in the laboratory.

All the subjects had been exposed for at least 4 months prior to the investigation. The half-times of the adducts in vivo are somewhat longer than 20 days (10), and, since it can be considered that it takes about three half-times to reach a steady state, it can be assumed that a steady state was reached.

The half-times of the adducts indicate that the levels of these adducts only show the exposure during the last month. However, in the work of Nielsen et al (7) current, as well as highest, ever exposure was assessed using air measurements. The correlations with the latter parameter did not show any closer associations than the current exposure, which indicated that there was no major selection bias due to any changes in the work-tasks.

Determination of specific antibodies

Specific antibodies of IgE and IgG were determined in serum from the workers, as previously reported (11). Briefly, the methods were as follows. The conjugates of HHPA and MHHPA were synthesized by the addition of the anhydrides to cooled solutions of HSA (37 mol anhydride/mol HSA) in 0.1 M sodium hydrogen

carbonate ($NaHCO_3$). An Amicon ultra filtration cell (8200, Amicon Corporation, Danvers, MA, USA) was used to purify the conjugates. The proteins were lyophilized and reconstituted in 0.1 M $NaHCO_3$ before use.

For the analysis of specific IgE the synthesized conjugates were bound to cyanogen-bromide-activated filter paper disks for use in a radioallergosorbent test (RAST) system (Phadebas, Pharmacia Diagnostics, Uppsala, Sweden). All the samples were analyzed in duplicate. The results were expressed as the percentage-specific binding (counts/minute of the test disc minus the counts/minute of the HSA reference disc) of the total added radioactivity. The sample was classified as positive if the value was above the highest value recorded for a referent.

The specific IgG was analyzed using enzyme-linked immunosorbent assay (ELISA). Polystyrene microtiter plates were coated with antigen solution and blocked for nonspecific binding. Thereafter, 100 µl of a 1:50 diluted (phosphate-buffered saline) solution of serum was added and incubated at 20°C for 60 minutes, 100 µl of an optimal dilution of alkaline phosphatase conjugated rabbit antihuman IgG (Dakopatts, Copenhagen, Denmark) was added and incubated at 20°C for 60 minutes, and 100 µl of substrate solution (disodium p-nitrophenol phosphate) was added and incubated at 20°C for 120 minutes. The results were read at 405 nm (Titertek Multiscan, Eflab, Helsinki, Finland). All the samples were analyzed in triplicate, and the results were expressed as the absorbance values. A value exceeding the highest value recorded for a referent was defined as positive.

Medical examination and atopy

Extensive occupational and medical histories, including smoking habits, were obtained from self-administered questionnaires and supplemented with interviews by a physician. Information about current and previous work-tasks at the present workplace, work-related symptoms, such as symptoms of the eyes (lacrimation, itching, scratching, smarting or burning), of the nose (blocked, runny, itchy or attacks of sneezing or bleeding), and of the lower airways (dyspnea, wheezing, chest tightness or dry cough) were collected. Symptoms were denoted as work-related if they appeared in relation to occupational activities and improved during weekends or holidays. Atopy was determined by a commercially available skin prick test with six common allergens from ALK laboratories (Copenhagen, Denmark).

Statistics

The prevalence odds ratio (POR) with its 95% confidence interval (95% CI) was used for measuring the effects of exposure on immunologic parameters and symptoms. The exposure variables were categorized into four similar-sized groups (table 1). As potential confounders, age (≥31 versus <31 years), gender, atopy, and smoking (smokers versus nonsmokers and ex-smokers) were considered. These variables were first tested, one at a time, for measuring the effects on the outcome variables. If the confounder indicated a univariate association with the outcome variable ($P<0.10$), we included the confounder together with the exposure variable in bivariate models. We present the adjusted effect estimates (in the text) if they differed from the crude estimates by >15%. In addition, by including interaction terms in the model, we tested whether these factors modified the effect of the exposure ($P<0.05$).

Table 1. Characteristics of the workers classified according to their HHPA and MHHPA exposures. (HHPA = hexahydrophthalic anhydride, MHHPA = methylhexahydrophthalic anhydride, TPPA = total plasma protein adducts)

	Corresponding HHPA air levels	Subjects			Age (years)		Smokers		Atopy	
		All	Women							
		(N)	N	%	Median	Range	N	%	N	%
TPPA of HHPA										
<40 fmol/ml	<1 µg/m³	35	15	43	29	20–61	9	26	9	26
40–100 fmol/ml	1–3 µg/m³	37	21	57	34	22–59	16	44	11	30
>100–300 fmol/ml	3–9 µg/m³	32	6	19	31	22–56	11	34	4	12
>300 fmol/ml	>9 µg/m³	35	7	20	33	21–64	9	26	7	20
Total		139	49	35	31	20–64	45	33	31	22
TPPA of MHHPA										
<100 fmol/ml	<1 µg/m³	33	23	70	36	20–64	13	40	5	15
100–300 fmol/ml	1–3 µg/m³	33	17	52	36	20–64	13	40	3	9
<300–1500 fmol/ml	3–15 µg/m³	35	9	26	29	21–53	11	31	10	29
>1500 fmol/ml	>15 µg/m³	38	0	0	29	22–52	8	21	13	34
Total		139	49	35	31	20–64	45	33	31	22

Results

The mean level of the total plasma protein adducts of HHPA was 840 (median 100, range <40–13800) fmol/ml, and the mean level of the total plasma protein adducts of MHHPA was 1700 (median 340, range <20–40300) fmol/ml. The approximate corresponding air levels calculated from the data of Rosqvist et al (10) and the characteristics of the subjects in the various exposure groups for HHPA and MHHPA have been tabulated in table 1. The ages of the subjects were similar in all the groups, but there seems to have been an overrepresentation of women in the lower exposure groups and also some differences in the frequency of atopics and smokers. There was no correlation between the total plasma protein adducts of HHPA and MHHPA (Spearman r=0.02, P=0.82).

Of all the workers, 19% was found to be positive for specific IgE, and 17–19% was positive for IgG. Positive associations were observed between the total plasma protein adducts of HHPA and specific IgE and IgG and between the total plasma protein adducts of MHHPA and specific IgG (table 2). For the association between the total plasma protein adducts of HHPA and specific IgG, the prevalence odds ratio increased somewhat when atopy was included in the model (adduct levels >300 fmol/ml versus adduct levels <40 fmol/ml, POR 15.5, 95% CI 3.0–80.6, not in table).

For the work-related symptoms, about 27% of the workers had symptoms of the nose, 21% had symptoms of the eyes, 11% reported symptoms of the lower airways, and 8% had nose bleeds. There was a positive association between HHPA exposure and symptoms of the eyes (table 3). Moreover, when gender was included in the model, the subjects with HHPA adduct levels above 300 fmol/ml had more frequent symptoms of the nose than the subjects with levels below 40 fmol/ml did (POR 3.4, 95% CI 1.0–11.1, not in table).

For the MHHPA exposure, there were no obvious exposure-response relationships with work-related symptoms (table 3).

Table 2. Associations between the TPPA of HHPA and MHHPA and respective specific IgE and IgG. (TPPA = total plasma protein adducts, HHPA = hexahydrophthalic anhydride, MHHPA = methylhexahydrophthalic anhydride, Ig = immunoglobulin, 95% CI = 95% confidence interval)

	Workers		Prevalence odds ratio	95% CI
	Total (N)	Sensitized (N)		
IgE				
TPPA of HHPA				
<40 fmol/ml	35	2	1.0	·
40–100 fmol/ml	37	7	3.8	0.7–20.0
>100–300 fmol/ml	32	8	5.5	1.1–28.2
>300 fmol/ml	35	10	6.6	1.3–32.8
TPPA of MHHPA				
<100 fmol/ml	33	8	1.0	·
100–300 fmol/ml	33	3	0.3	0.1–1.3
>300–1500 fmol/ml	35	7	0.8	0.2–2.5
>1500 fmol/ml	38	9	1.0	0.3–2.9
IgG				
TPPA of HHPA				
<40 fmol/ml	35	2	1.0	·
40–100 fmol/ml	37	1	0.5	0.04– 5.3
>100–300 fmol/ml	32	6	3.8	0.7 –20.4
>300 fmol/ml	35	15	12.4	2.6 –59.9
TPPA of MHHPA				
<100 fmol/ml	33	4	1.0	·
100–300 fmol/ml	33	4	1.0	0.2– 4.4
>300–1500 fmol/ml	35	8	2.1	0.6– 8.0
>1500 fmol/ml	38	11	3.0	0.8–10.4

Discussion

The results of our study showed that there was an exposure-response relationship for HHPA both with specific antibodies and with work-related symptoms. In addition, the results elucidate the potential power of total plasma protein adducts as relevant indices of exposure and risk.

There are only very few scientific reports on exposure-response relationships for allergens (12). The absence of such information has, in the past, led to an assumption that such relationships do not exist. One explanation for the lack of any exposure-response relationships that has been discussed is the risk of selection bias in cross-sectional studies. Reliable methods for high-quality exposure assessment are another requirement for successful studies. Thus, recently, when new immunologic methods have been applied for quantifying exposure to protein allergens in large study groups, nice exposure-response relationship assessments for, for example, α-amylase (13, 14) and urinary rat allergens (15) were obtained.

For low-molecular-weight chemicals that must conjugate to endogenous proteins to form full antigens, the monitoring of exposure has been possible through the air analysis of the low-molecular-weight chemical itself. However, extended air monitorings are very laborious, and, since exposure tends to vary a lot in chemical plants, such exposure assessments are somewhat crude. As a probable consequence, any demonstrated exposure-response relationships for low-molecular-weight sensitizers are also very sparse. In this report we have used the total plasma protein adducts of HHPA and MHHPA as indices of the exposure. These biomarkers reflect the exposure during the last couple of months.

Nielsen et al (7) reported relationships between work-related symptoms and specific antibodies from the same group of subjects included in our present study. However, in that study, the exposure to HHPA and MHHPA was quantified using air measurements and the biological monitoring of metabolites in urine. The urine samples were collected on one single occasion in conjunction with the medical examination and thus showed only the single-day exposure. When the workers were divided into different groups according to their exposure, as determined as air or adduct levels, the associations with specific IgE were stronger in our present study. For specific IgG, there was an association with the exposure in both studies. There were also similar associations between all the exposure indices and work-related symptoms in both studies. However, the estimated exposure levels giving rise to specific antibodies or symptoms in the present study seem to be lower than those of the previous study. On the other hand, this finding may be a consequence of the division into exposure groups, for which, in the former study, the lowest exposure group was chosen to be <10 $\mu g/m^3$, a value considerably higher than that of our present study.

Recently Welinder et al (6) published a study showing a clear exposure-response relationship for OAA-specific IgE and also for IgG. The OAA studied were HHPA, MHHPA, and methyltetrahydrophthalic anhydride, and the exposure assessment was based on the sum of all three OAA. It is interesting to note that there is a similarity with the present study both regarding the levels of exposure giving rise to sensitization and the picture that the exposure threshold value for the production of IgE antibodies is lower than for IgG antibodies. The study of Welinder et al (6) was a prospective study and, therefore, could better estimate the exposure than our cross-sectional study since the exposures in that study were followed during the whole exposure period. On the other hand, Welinder and his co-workers (6) used ambient monitoring for exposure assessments, and, although air measurements were taken many times, they represented only a very small fraction of the real exposure time.

In our study, when the workers were divided into four exposure categories, the levels of the MHHPA adducts in each of the groups were higher than the levels of the corresponding HHPA adducts. However, for a given air exposure, the levels of MHHPA adducts are about three times higher than for HHPA (10). Thus, although the levels of the total plasma protein adducts of MHHPA are higher than those of HHPA, the air exposure levels of the anhydrides are almost the same within the various exposure groups.

The work areas with the highest exposures to HHPA and MHHPA were the ones with the heaviest workload.

Table 3. Associations of the TPPA of HHPA and MHHPA with work related symptoms. (TPPA = total plasma protein adducts, HHPA = hexahydrophthalic anhydride, MHHPA = methylhexahydrophthalic anhydride, 95% CI = 95% confidence interval, NA = not possible to estimate.)

Work related symptoms	Workers		Prevalence odds ratio	95% CI
	Total (N)	Sensitized (N)		
Lower airways				
TPPA of HHPA				
<40 fmol/ml	35	4	NA	·
40–100 fmol/ml	37	3	NA	·
>100–300 fmol/ml	32	0	NA	·
>300 fmol/ml	35	8	NA	·
TPPA of MHHPA				
<100 fmol/ml	33	3	1.0	·
100–300 fmol/ml	33	6	2.2	0.5–5.8
>300–1500 fmol/ml	35	5	1.7	0.4–7.6
>1500 fmol/ml	38	1	0.3	0.1–2.7
Eyes				
TPPA of HHPA				
<40 fmol/ml	35	3	1.0	·
40–100 fmol/ml	37	7	2.5	0.6–10.5
>100–300 fmol/ml	32	7	3.0	0.7–12.7
>300 fmol/ml	35	12	5.6	1.4–22.0
TPPA of MHHPA				
<100 fmol/ml	33	8	1.0	·
100–300 fmol/ml	33	7	0.8	0.3–2.7
>300–1500 fmol/ml	35	6	0.6	0.2–2.1
>1500 fmol/ml	38	8	0.8	0.3–2.5
Nose				
TPPA of HHPA				
<40 fmol/ml	35	6	1.0	·
40–100 fmol/ml	37	10	1.8	0.6–5.6
>100–300 fmol/ml	32	10	2.2	0.7–7.0
>300 fmol/ml	35	12	2.5	0.8–7.7
TPPA of MHHPA				
<100 fmol/ml	33	12	1.0	·
100–300 fmol/ml	33	9	0.7	0.2–1.9
>300–1500 fmol/ml	35	8	0.5	0.2–1.5
>1500 fmol/ml	38	9	0.5	0.2–1.5
Nose bleed				
TPPA of HHPA				
<40 fmol/ml	35	1	1.0	·
40–100 fmol/ml	37	3	3.0	0.3–30.3
>100–300 fmol/ml	32	2	2.3	0.2–26.3
>300 fmol/ml	35	5	5.7	0.6–51.3
TPPA of MHHPA				
<100 fmol/ml	33	3	1.0	·
100–300 fmol/ml	33	1	0.3	0.1–3.2
>300–1500 fmol/ml	35	4	1.3	0.3–6.3
>1500 fmol/ml	38	3	0.9	0.2–4.6

Thus a higher proportion of female employees was found in the lower exposure groups.

While the total plasma protein adducts of HHPA seem to give strong associations with specific antibodies and work-related symptoms, for the total plasma protein adducts of MHHPA, a dose-response relationship was evident only with specific IgG. The associations became weaker when the sum of the HHPA and MHHPA adduct levels correlated with antibodies or

work-related symptoms compared with HHPA only (results not shown). Thus there are reasons to believe that HHPA may be more sensitizing than MHHPA. However, there are some alternative explanations for this observation. For example, a co-exposure to HHPA could mask the effects of MHHPA. Since there is a strong cross-reactivity between HHPA and MHHPA antibodies, the test does not differentiate between them. There was no correlation between the total plasma protein adducts of HHPA and MHHPA, but a closer look at the workers positive for specific IgE in the lowest MHHPA-exposure group identifies them as mainly HHPA-exposed, half of them in the highest HHPA exposure group. However, this finding still does not explain the lack of association between MHHPA exposure and work-related symptoms in all four exposure groups. Another explanation for the lack of exposure-response relationships for MHHPA could be that this anhydride is so potent that even the lowest exposed workers in this study are at high risk of sensitization. An observation that could support this explanation may be presented. Thus, for HHPA, the threshold for the induction of IgE seems to be lower than that of IgG, a finding in agreement with the data from Welinder et al (6). In addition, the induction of IgG for MHHPA seems to start at a lower level than for IgG for HHPA. According to this reasoning, the threshold for IgE response for MHHPA may well be below that of IgG for MHHPA (ie, at levels below the monitored exposure levels of this study).

In studies of natural protein allergens, air levels in the low nanogram per cubic meter region seem to induce the production of specific IgE (eg, rat urinary protein, α-amylase or latex) (13–16). In this study of two low-molecular-weight chemicals, the levels causing sensitization were three orders of magnitude higher. However, it has been reported that the majority of inhaled OAA are hydrolyzed to the corresponding acids, which are rapidly excreted through urine (17), and that probably less than 1% binds to proteins. Thus these low-molecular-weight chemicals seem to be allergens that are nearly as potent as naturally occurring proteins.

Studies on exposure-response relationships are important for establishing causality and occupational exposure limits. Such studies often demand the analysis of a vast number of samples with accurate and reliable assessment of the exposure intensity. The analysis of protein adducts has the potential of monitoring long-term exposure, but it has only been sparingly used in exposure-response studies to date. The analytical method we used in this study was simple, easy, and practical, and it allowed the analysis of a large number of samples with a detection limit sufficient to monitor very low exposure levels. The good detection limits is particularly important when the fact that sensitization occurred at exposure levels of only a few micrograms per cubic meter is considered. Thus earlier studies on exposure-response relationships of HHPA and MHHPA suggest an occupational exposure limit of less than 10–20 $\mu g/m^3$ (7). This was an air level that has been found to prevent symptoms in IgE-sensitized workers, but the limit was insufficient for preventing sensitization. However, the results of our study, as well as data from Welinder et al (6), suggest that this value is even lower. We found evident exposure-response relationships with both symptoms and specific IgE down to protein HHPA-adduct levels of 40 fmol/ml, corresponding to air levels of about 1 $\mu g/m^3$. In addition, in the lowest exposed group, the prevalence of specific IgE was 6%, while that of symptoms of the eyes and nose was 9% and 17%, respectively. These values could have been influenced by exposure to MHHPA and the presence of background levels of symptoms in the normal population; the corresponding levels of work-related symptoms in the control group were 14% and 16% for the eyes and nose, respectively (7). However, in the second lowest exposure group in this study, the prevalence of specific IgE was 19%, and that of eye and nose symptoms was 19% and 27%, respectively, which must be considered to be unacceptably high.

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