Protein adducts in plasma as biomarkers of exposure to and risk of organic acid anhydrides

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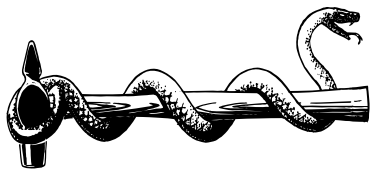

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Seema Bairoliya Rosqvist

AKADEMISK AVHANDLING
Som med vederbörligt tillstånd av medicinska fakulteten vid Lunds universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligen försvaras i föreläsningssal F1, Universitetssjukhuset i Lund
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Fakultetsopponent: Professor Dr. Hans Drexler, Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine,
University of Erlangen-Nürenberg, Germany

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Abstract

Title
Protein adducts in plasma as biomarkers of exposure to and risk of organic acid anhydrides

Organic acid anhydrides (OAAs) are important industrial chemicals but also potent inducers of airways diseases. Many exposed workers develop specific IgE and IgG antibodies. Hexahydrophthalic anhydride (HHPA) and methylhexahydrophthalic anhydride (MHHPA) are two particularly sensitising OAAs. The aim of this thesis was to develop and evaluate a method for biological monitoring of exposure to and risk of HHPA and MHHPA, using protein adducts in plasma as biomarkers. A total of 141 workers exposed to HHPA and MHHPA and two guinea pigs exposed to HHPA were investigated. Plasma from these were dialysed and the total plasma protein adducts (TPPA) of the anhydrides were hydrolysed. The acids thus formed were extracted, derivatised and analysed by gas chromatography-mass spectrometry (GC-MS). Air levels and urinary metabolite levels of the anhydrides were also analysed by GC methods. Chromatography, electrophoresis and immunological methods were used for separation, identification and quantification of proteins and Igs. Medical histories and symptoms were assessed through questionnaires supplemented with interviews and medical examinations. A quick, simple, accurate, precise and sensitive quantification method for TPPA of OAAs was developed. There were very strong correlations between long-term exposures, as assessed by repeatedly collected urine samples and hemoglobin adducts, and TPPA. Serum albumin (SA) was found to be the major OAA adduct forming protein. Anhydride specific IgE and IgG showed a preferred affinity for SA adducts. There were significant exposure-response relationships for TPPA of HHPA with specific IgE and IgG and symptoms and for TPPA of MHHPA with specific IgG. Thus, the TPPA can be considered to be excellent biomarkers of long-term exposure and of risk. A biological occupational exposure limit for HHPA and MHHPA is suggested.

Key words

Hexahydrophthalic anhydride, methylhexahydrophthalic anhydride, biological monitoring, serum albumin, exposure-response, IgE, IgG, GC-MS, OEL

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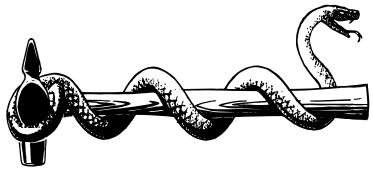

Protein adducts in plasma as biomarkers of exposure to and risk of organic acid anhydrides

Seema Bairoliya Rosqvist

DOCTORAL THESIS
Disputaion: Thursday 11th October 2001 at 13.15
Lecturehall F1, Centralblock, Lund University Hospital, Lund, Sweden.
Faculty Opponent: Professor Dr. Hans Drexler, Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine,
University of Erlangen-Nürenberg, Germany

FACULTY OF MEDICINE
University of Lund

2001

Hi Mum
Grandad used to say *"Jack of all trades*
- Master of none"

And now *"- Doctor of some"*

Lots of love, Seema XXX.

"Dare to walk the untrodden path; leave a litter of smile and wisdom behind…."

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Stora mängder reaktiva lågmolekylära ämnen används i den kemiska industrin vid produktionen av varor som hanteras i vårt dagliga liv. Exempel på sådana kemikalier är organiska syraanhydrider (OAA). En stor andel av de arbetare som exponeras för dessa viktiga industriella föreningar blir tyvärr sjuka med symtom som astma och hösnuveliknande besvär. I åtminstone en del fall är sjukdomen allergiskt betingad. För att effektivt skydda arbetarna måste man veta vilka exponeringsnivåer som ger upphov till sjukdom, det vill säga vad som vanligen brukar benämnas som exponerings-responssamband. För två OAA, hexahydroftalsyra-anhydrid (HHPA) och metylhexahydroftalsyra-anhydrid (MHHPA), som främst används för framställning av elektroniska komponenter, finns mycket lite sådan data. Detta förhindrar att ett hygieniskt gränsvärde kan tas fram.

Målet med den här studien var att utveckla en metod för att mäta exponeringen för HHPA och MHHPA samt att med hjälp av denna ta fram exponerings-responssamband för dessa anhydrider.

Den metod som utvecklades för att mäta exponeringen baseras på analys av de anhydrider som bundit till protein i plasman hos arbetarna, så kallade proteinaddukter. Metoden använder sig av en avancerad och mycket känslig analysmetodik som kallas gaskromatografimasspektrometri. Det visas i avhandlingen att halten av proteinaddukterna är ett bra mått på exponeringen för anhydrid under mer än en månad tillbaka och att serumalbumin är det protein i plasma som huvudsakligen bildar addukter med syraanhydriderna. Vidare insamlas i avhandlingen plasma från 141 arbetare som exponeras för HHPA och MHHPA och halterna av protein-addukter bestäms i dessa. Utöver detta studeras de arbetsrelaterade symptom arbetarna har samt halterna av antikroppar specifika mot anhydriderna vilket kan vara ett tecken på allergisk sjukdom. Halterna av syraanhydridaddukter relateras sedan till antikroppsnivåerna och symtom varvid det visade sig att även en ytterst låg exponering för åtminstone HHPA ökar risken för att utveckla sjukdom betydligt. Slutligen lämnas ett förslag till biologiskt gränsvärde för anhydriderna.

LIST OF PAPERS

This thesis is based upon the following papers, referred to by their Roman numerals:

I **Rosqvist S.**, Johannesson G., Lindh C. H. and Jönsson B. A. G. (2000) Quantification of protein adducts of hexahydrophthalic anhydride and methylhexahydrophthalic anhydride in human plasma. *J. Environ. Monitor.*, **2**, 155-60.

II **Rosqvist S.**, Johannesson G., Lindh C. H. and Jönsson B. A. G. (2001) Total plasma protein adducts of allergenic hexahydrophthalic and methylhexahydrophthalic anhydrides as biomarkers of long-term exposure. *Scand. J. Work Environ. Health*, **27**, 133-139.

III Johannesson G., **Rosqvist S.**, Lindh C. H., Welinder H. and Jönsson B. A. G. (2001) Serum albumins are the major site for *in vivo* formation of hapten-carrier protein adducts in the plasma from humans and guinea-pigs exposed to type-1 allergy inducing organic acid anhydrides. *Clin. Exp. Allergy*, **31**, 1021-1030.

IV **Rosqvist S.**, Nielsen J., Welinder H., Rylander L., Lindh C. H. and Jönsson B. A. G. Exposure-response relationships for hexahydrophthalic and methylhexahydrophthalic anhydrides using total plasma protein adducts as biomarkers. (manuscript).

CONTENTS

ABBREVIATIONS

CI	Confidence interval
CPM	Counts per minute
CV	Column volume
DMBA	N,N-dimethylbenzylamine
FEV_1	Forced expiratory volume in 1 second
FID	Flame ionisation detection
FVC	Forced vital capacity
GC-MS	Gas chromatography mass spectrometry
Hb	Hemoglobin
[$^3H_2$] HHPA	Tritium labelled hexahydrophthalic anhydride
HHPA	Hexahydrophthalic anhydride
HHP	Hexahydrophthalic
HSA	Human serum albumin
IEC	Ion exchange chromatography
IgE	Immunoglobulin E
IgG	Immunoglobulin G
LC	Liquid chromatography
LMW	Low molecular weight
MHHPA	Methylhexahydrophthalic anhydride
MHHP	Methylhexahydrophthalic
MTHPA	Methyltetrahexahydrophthalic anhydride
NICI	Negative ion chemical ionisation
OEL	Occupational exposure limit
OAA	Organic acid anhydride
PBS	Phosphate buffered saline
PEG	Polyethylene glycol (molecular weight 20,000)
PFBBr	Pentafluorobenzyl bromide
PFB-HHP	Pentafluorobenzyl hexahydrophthaloate
PFB-MHHP	Pentafluorobenzyl methylhexahydrophthaloate
POR	Prevalence odds ratio
r	Linear regression correlation coefficient
r_s	Spearman`s rank correlation coefficient
SA	Serum albumin
SPT	Skin prick test
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPE	Solid phase extraction
SIM	Selected ion monitoring
TBST	Tris buffer saline tween
TPPA	Total plasma protein adducts
WRS	Work-related symptoms

INTRODUCTION

General Background

Reactive, low molecular weight (LMW) compounds are widely used in the industry, in the production of many everyday products and articles. Due to their reactivity, many of these compounds are also capable of causing adverse health effects such as airways and skin diseases and cancer in exposed workers. The organic acid anhydrides (OAAs) are an example of such useful but also harmful compounds.

Organic acid anhydrides

OAAs are used extensively as crosslinking agents in the production of a variety of products ranging from electronic components, pesticides, pharmaceuticals, synthetic fibres and textiles, furniture, paints, adhesives dyes, and plastic packaging materials such as polyvinyl chloride. Vast quantities are used in the USA and Europe every year. Some 500 thousand tons of phthalic anhydride and 150 tons of maleic anhydride were used only in the European Union in 1998. However, OAAs are also known to cause adverse health effects in exposed workers, primarily through sensitisation of the airways and the eyes.

Hexahydrophthalic anhydride (HHPA) and methylhexahydrophthalic anhydride (MHHPA), the two OAAs studied in this thesis, are commonly used as hardeners in production of epoxy resins (**Figure 1**). These resins are mainly used in manufacture of components for electrical appliances. In 1991, the amount of HHPA used in the Swedish industry was estimated to be around 200 tons (Jönsson 1992). HHPA and MHHPA are particularly sensitising compounds (Welinder *et al*. 1994, Welinder *et al*. 2001, Nielsen *et al*., in press) but no occupational exposure limit (OEL) has been set in Sweden or elsewhere hitherto. This is because information on exposure-response relationships of the OAAs is very scanty. In order to clarify this situation, reliable methods for exposure assessment must be developed. These are essential in evaluation of such relationships and thus in establishment of OELs.

Chemical and physical properties of HHPA and MHHPA

HHPA exists in two isomeric forms, *trans* and *cis* with respect to the carboxlic groups. The *cis*-isomer with the chemical abstract service (CAS) number 13149-00-3 is studied in this thesis. The isomer of MHHPA (CAS number 25550-51-0) investigated in this work is also *cis*

with respect to the carboxylic group but a mixture of *cis/trans* (50:50) with respect to the methyl group on the hexane ring.

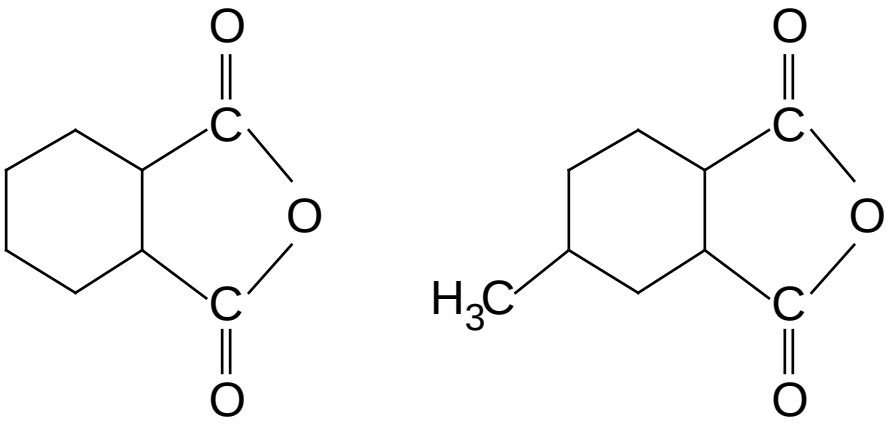

Figure 1. Chemical structures of HHPA and MHHPA

OAAs are spontaneously hydrolysed in water to form the corresponding acids. HHPA hydrolyses with a half-time of about 0.2 min (Eberson and Landström, 1972) to hexahydrophthalic acid (HHP acid). There is no published data available on the rate of hydrolysis of MHHPA to methylhexahydrophthalic acid (MHHP acid) but its behaviour can be assumed to be similar to HHPA. The anhydrides also react readily with nucleophiles such as amino and thiol groups to form amides and thiol esters, respectively. Some physical properties are listed in **Table 1**.

Table 1. Physical properties of HHPA and MHHPA.

Properties	**HHPA**	**MHHPA**
Appearance	White Crystals	Colourless, oily-liquid
Molecular Weight	154	168
Melting Point	35°C	-29°C
Boiling Point	270°C	120°C at 130 Pa
Equilibrium Vapour Pressure	50 Pa at 20°C	NA[a]

[a]NA=no data available

Biological effects of HHPA and MHHPA

The major clinical effects from exposures to HHPA and MHHPA seem to be hypersensitive reactions giving symptoms from the eyes and the upper and lower airways. Symptoms reported include immediate/late asthma, conjunctivitis, rhinitis, nose-bleed and skin reactions. (Moller *et al*. 1985, Venables, 1989, Drexler *et al*. 1994, Grammer *et al*. 1996, Drexler *et al*. 1999, Kanerva *et al*. 1999, Nielsen *et al*., in press). The pathomechanisms behind the development of these diseases are not clear. Anhydride specific immunoglobulin (Ig)E and IgG antibodies have been found in a large proportion of workers exposed to HHPA and/or MHHPA (Moller *et al*. 1985, Drexler *et al*. 1994, Welinder *et al*. 1994, Drexler *et al*. 1999 and 2000, Welinder *et al*. 2001, Nielsen *et al*., in press).

The relevance of the IgE antibodies for induction of airways symptoms have been proved by specific nasal challenges using a conjugate between HHPA and human serum albumin (HSA; Nielsen *et al*. 1994). This suggests one of the pathomechanisms, in at least some of the symptomatic workers, to be an IgE mediated Type-1 allergy as classified by Coombs and Gell in 1963. Thus, the conjugation of anhydrides with endogenous proteins can be considered to be an important step in this pathomechanism. This is further supported by the

fact that the non-reactive acids from hydrolysis of the anhydrides do not seem to elicit any immunological responses (Zhang 1997).

The pathogenic relevance of the specific IgG is more diffuse and obscure. Nasal challenge studies carried out by Nielsen *et al*. (1994) showed a lack of any associations between nasal symptoms and specific IgG in HHPA exposed workers. This suggests that the presence of IgG may just show that there has been an exposure to anhydride; IgG do not seem to play a role in the development of the symptoms. On the other hand, Grammer and co-workers (1993, 1994, 1996a and 1996b) have suggested that the specific IgG is associated with nose-bleed. However, in a recent work, the nose-bleedings in HHPA and MHHPA exposed workers were associated with high levels of both, specific IgE and IgG (Nielsen *et al*. in press).

A non-immune response due to the direct toxic effects of the anhydrides may also occur (Bernstein and Bernstein, 1994). Although animal studies and also experimental exposures of 8 hours to human volunteers did not seem to induce any direct toxic effects from HHPA (BAG Jönsson and CH Lindh, personal communication).

There are very few reports on exposure-response relationships for HHPA and MHHPA. For HHPA Grammer *et al*. (1994) found that workers who were positive for specific IgE had had a higher exposure than those tested negative. However, the exposures were not measured but only estimated by a senior management chemical engineer. Welinder *et al*. (2001) found exposure-response relationships between the sum of the air levels of HHPA, MHHPA and methyltetrahydrophthalic anhydride (MTHPA) and OAA-specific IgE, and IgG in a prospective study. In a cross sectional study Nielsen *et al*. (in press) reported exposure-response relationships between the sum of the air levels of HHPA and MHHPA, and specific antibodies and also work related symptoms (WRS). In the same study exposure-response relationships were also found between the exposure, as estimated by urinary metabolites, and specific antibodies and WRS. However, for accurate and conclusive estimations of risk and also for establishment of OELs more studies are needed.

Metabolism of HHPA and MHHPA

Occupational exposure to HHPA and MHHPA is mainly via inhalation of vapours and to a lesser degree through skin. Studies using controlled human exposures have shown that inhaled HHPA is almost completely absorbed through the mucous membrane in the respiratory tract (Jönsson

and Skerfving 1993). After entering the body, most of the anhydride is rapidly hydrolysed to HHP acid and distributed to the blood and the extracellular fluids. The levels of the acids in plasma and urine were found to increase at exposure and then decay after the end of exposure with a half-time of about 2 hours. There are also other reported elimination half-times in urine from exposed workers which vary from 5 up to 14 hours (Pfäffli *et al*. 1989, Jönsson and Skerfving 1993, Jönsson *et al*. 1993). The acids were found to be almost completely excreted through the kidneys, in unconjugated form, by a mechanism relatively unaffected by urinary pH. Studies on workers exposed to MHHPA show similar results (Pfäffli *et al*. 1989, Lindh *et al*. 1997).

With their autoradiography studies Lindh *et al.* (1999) showed that following one single inhalation exposure of guinea-pigs and rats to tritium-labelled HHPA, covalently adducted HHPA was found predominantly in the upper respiratory tract, trachea and the conjunctiva. The lungs did not seem to contain any non-extractable radioactivity. Labelling was seen in the mucosa of the mouth, upper alimentary tract, blood proteins and also some distal tissues, such as kidneys in rats were labelled. There was no labelling observed in guinea-pig kidney, suggesting a species difference in distribution mechanism. The radioactivity in the tissues persisted for at least seven days after the end of exposure. The major HHPA adduct forming protein in human erythrocytes seemed to be hemoglobin (Hb) and this study also suspected serum albumin (SA) to be the major HHPA adduct site in plasma.

In exposed workers, it has been shown that a minor part of the inhaled HHPA and MHHPA form adducts with Hb (Lindh and Jönsson, 1998b). These adducts were found to be stable *in vivo*. Furthermore, lysine adducts in collagen of guinea-pig lung after exposure to MTHPA has been reported (Jönsson *et al*. 1995). However, there is still very little information on protein adducts of OAAs. Such information is important in several ways. Since type-I allergy is one pathomechanism behind anhydride induced airways disease, the binding to endogenous proteins is the first step in the sensitisation process. Thus, identification of the proteins that bind to anhydride will provide important information on the mechanism behind the induction of disease.

Furthermore, in the tests for OAA specific antibodies, SA has been routinely used as the carrier protein. It is therefore possible that these tests will miss detection of antibodies directed against protein adducts other than those of SA. Also, as described later in this thesis,

protein adducts have a potential to be excellent biomarkers of exposure and risk.

Assessment of exposure to HHPA and MHHPA

Air measurements

There are methods described for air monitoring of HHPA and MHHPA (Pfäffli *et al*. 1989, Jönsson *et al*. 1991 and 1996). These methods use bubblers, impingers, filter or solid sorbent tubes such as Amberlite XAD-2 or Tenax, to trap the OAAs. Gas chromatography (GC) with flame ionisation detection (FID), electron capture detection or mass spectrometry (MS) are used for detection and quantification. Air monitoring is useful in assessment of peak exposures and emission sources. It is useful in the cases where data on direct and specific exposure is required. For example, in assessing symptoms from the eyes, it is the dose deposited directly on the eyes that is relevant rather than the integrated exposure as given by biological monitoring.

Biological monitoring

Biological monitoring assesses the internal or effective exposure/dose, *i.e*. the dose at the target sites. It shows the actual uptake by the workers, which is effected by many factors such as variability in work load, various routes of uptake, use of protective devices, personal hygiene habits and non-occupational exposure. It has a potential to account for individual health impairments and differences in metabolic rates. Thus, it may be more directly related to the overall adverse health effects and a better estimate of comprehensive health risk. Biological sampling also tends to be easier, cheaper and more convenient compared to air sampling. In addition, biological sampling is useful in "post-event" situations such as monitoring after an unplanned or accidental exposure.

There are methods described for biological monitoring of HHPA (Jönsson *et al*. 1991 and 1993, Jönsson and Skerfving 1993) and MHHPA (Lindh *et al*. 1997) using metabolites in urine and plasma. These methods are based on quantification of the corresponding acids from the parent anhydride. The levels of acids correlate strongly with levels of the anhydrides calculated from air monitorings. Most of these methods work well with good precision and acceptable detection limits, reflecting absorbed dose in workers but due to the short elimination half-time of the acids in urine and plasma, they monitor only the short-term exposure, typically exposure from one work shift. Multiple or repetitive sampling and analysis over extended periods is required to measure long-term exposure.

Alternatively, long-term exposure may be estimated through the biological monitoring using long-lived biomarkers, such as protein adducts. The use of protein adducts as indices of exposure to toxic compounds was first suggested in 1974 by Ehrenberg *et al*. The idea is that a relatively long-lived protein-hapten conjugate of a xenobiotic could work as a dosimeter. The concept has been developed over the years and has found its use for example in the biomonitoring of aromatic amines (Bryant *et al*. 1988), polycyclic aromatic hydrocarbons (Skipper *et al*. 1984) and acrylamide (Calleman *et al*. 1994) but its full potential is still to be exploited. The few hitherto publications on protein adducts of OAAs describe Hb-adducts of HHPA and MHHPA as suitable biomarkers of long-term exposure (Jönsson *et al*. 1997; Lindh and Jönsson 1998a and 1998b). However, the extent of adduction to plasma proteins are normally higher than those to Hb (Sepai *et al*. 1995, Yeowell O´Connell *et al*. 1998) giving the possibility to develop methods that are more sensitive. Moreover, methods based on the analysis of total plasma protein adducts (TPPA) have a potential of being practical and simple. Thus, a method for biological monitoring of HHPA and MHHPA using TPPA should be developed and evaluated. Such an evaluation should include comparison of exposures as quantified using other long-term exposure assessment methods as well as toxicokinetic studies of the protein adducts.

AIM OF THE THESIS

General aim

To develop and evaluate a method for biological monitoring of exposure to and risk of HHPA and MHHPA, using protein adducts in plasma as biomarkers

Specific aims

To develop and validate a method for quantification of TPPA of HHPA and MHHPA

To elucidate the toxicokinetics of TPPA of HHPA and MHHPA

To evaluate the applicability of TPPA as biomarkers of exposure to HHPA and MHHPA

To identify proteins in plasma that form adducts with HHPA and MHHPA

To investigate whether plasma protein-HHPA conjugates have affinity for IgE- and IgG-antibodies from exposed workers

To establish exposure-response relationships and to evaluate the applicability of TPPA as biomarkers of risk of HHPA and MHHPA

MATERIALS AND METHODS

The plant

All workers studied in this thesis were employed at a plant manufacturing electrical capacitors. The capacitors were insulated and mechanically fixed using an epoxy resin with HHPA and MHHPA as hardeners. The air levels of HHPA and MHHPA in the plant, as monitored hrough personal sampling, were up to 400 µg HHPA/m^3 and 200 µg MHHPA/m^3. These values are lower than the equilibrium vapour pressure concentration, thus the major exposure was assumed to be in vapour phase (Jönsson *et al*. 1993). The OAAs were handled primarily in closed systems or ventilated hoods, but some manual operations were performed with uncured HHPA and MHHPA at elevated temperatures (approx. 60-80°C). Thus, the main exposures occurred during casting, leakage from curing ovens and during the transfer of hot components from the ovens to other departments. All workers had been exposed for at least four months prior to the investigation. Epichlorohydrin based epoxy resin, dimethylbenzylamine (DMBA) and acetone were also used in the manufacturing process.

Workers

In 1994, the workforce in the plant was invited to participate in an extensive study on exposures to HHPA and MHHPA and their health effects. Of the 157 invited workers, a total of 154 participated in the various investigations (Nielsen *et al*. in press). The major part of this particular study involved 139 of the above workers, with a median age of 31 (range 20-64), 49 (35%) were women, 45 (33%) were smokers and 21 (15%) were classified as atopics by history.

Samples from workers

Plasma samples for adduct determinations were obtained from 141 out of the 154 subjects **(Paper IV)**. Serum was obtained from 139 of these. Urine was sampled from 117 of these 139 workers, the same week as the blood was sampled **(Paper II;** result for one MHHPA exposed worker missing). The plasma from some of these workers were used in **Paper I** to study hydrolysis, precision, correlations between TPPA and SA adducts of MHHPA and between air levels of OAA and TPPA levels.

Furthermore, in a study by Jönsson *et al*. (1997) the long-term exposure to HHPA and MHHPA was assessed through analysis of urine

samples, collected repeatedly, from 10 of these workers. The urine was sampled during the last four hours of a work-shift, on ten to twelve different days, over a period of four weeks. Corresponding blood samples were taken at the end of the 4 week period. and were used for determination of Hb adducts of HHPA (Jönsson *et al*. 1997), SA adducts and TPPA of HHPA **(Paper I)** and TPPA of both HHPA and MHHPA **(Paper II)**. These blood samples were collected in addition and on a separate occasion than the 141 samples collected above. The plasma from one of these workers was also used for the characterisation of plasma protein adducts in **Paper III** (subject 6).

For toxicokinetics studies **(Paper II and new results)**, blood was collected before and after a 28 to 35-day period without exposure, from 4 workers exposed to both HHPA and MHHPA and 3 workers mainly exposed to HHPA (TPPA of MHHPA below detection limit). Only four of these workers were the same as the five analysed for Hb-adducts by Lindh *et al*. (1998b) because plasma was missing from one of the workers. In addition, one determination of TPPA of HHPA from a worker also reported by Lindh *et al*. (1998b) was excluded because of inconclusive results when the plasma was finished. Five of the workers were from the 141 workers investigated in **Paper IV** but the samples were collected on a separate occasion.

Blood was also collected from one HHPA exposed and one MHHPA exposed worker who ended employment and were thereafter unexposed. Blood samples from these two workers were collected on the last day of work and then once a month over four months after the termination of employment. These workers were also from the 141 investigated in **Paper IV** but again the samples were collected on a separate occasion.

For determination of detection limits of the quantification method (**Paper I**) blood was sampled from 10 personnel in our laboratory.

Sera were collected from 3 workers with high titers of HHPA specific IgG (**Paper III**; subjects 1-3) and from 2 workers with high HHPA specific IgE (**Paper III**; subjects 4 and 5) Sera were also obtained from a total of 57 control subjects from two mechanical industries (**Paper IV**).

Guinea-pigs

White, inbred male guinea-pigs of Dunkin-Hartley strain, weighing about 350 g, (Sahlins) were acclimatised for one week and then maintained on the facilities, with food and water provided *ad libitum* on

a cycle of 8 hours light and 16 hours dark. During exposure, the animals had access to water only (**Paper III**).

Exposure of guinea-pigs to HHPA

A guinea-pig was exposed to unlabelled HHPA at an air level of 1300 µg/m^3 in a 70 l glass chamber for 5 hours/day, 5 days/week for 2 weeks (**Paper III**). The gaseous HHPA was generated by a permeation technique as previously described (Jönsson *et al*. 1994). Immediately after the last exposure the animal was anaesthetised by an intra-muscular injection (1 ml/kg body weight) of a mixture of ketamine hydrochloride (50 mg/ml; Park-Davis) and xylazine chloride (20 mg/ml; Bayer), prepared in a ratio of 3:2. After collection of blood the animal was killed by an overdose of ketamine/xylazine.

The procedure for exposure of a guinea-pig to radioactive HHPA has been previously described (Lindh et al. 1999). The exposure level was 500 µg/m^3 (3 µmol HHPA/m^3) with a specific activity of 34 GBq/µg (0.2 GBq/µmol) HHPA.

Collection of biological samples

All human blood samples were drawn from an antecubital vein. Blood for plasma and Hb was collected in 10 ml Venoject® blood sampling tubes (Terumo Europe) containing sodium heparin (**Paper I-IV**). Blood from the guinea-pigs was collected by heart puncture in tubes containing sodium heparin (**Paper III**). After sampling, the blood was allowed to cool to room temperature and then centrifuged at 1500*g* for 10 min for the separation of plasma. All samples were stored in polyethylene test tubes at -20°C until analysis.

For collection of sera, the blood samples were collected in glass test tubes and allowed to clot at room temperature for one hour (**Paper III and IV**). The tubes were stored at 4°C overnight after which they were centrifuged and the sera separated. Urine was sampled during the last 4 hours of an 8 hour work-shift and stored in polyethylene test tubes at -20°C until analysis.

Ethics

Permission for the human study was obtained from the ethics committee at Lund University. The animal study was approved by the Animal Research Ethical Committee at Lund University.

Determination of HHPA and MHHPA in air

In **Paper I**, the air levels of OAA were determined by personal air sampling using Amberlite XAD-2 sorbent tubes as previously described (Jönsson *et al*. 1996a). The samples were stored at -20°C and analysed within a week after sampling. The rapped OAAs were extracted from the XAD-2 with toluene and analysed by GC-FID.

Determination of HHP acid and MHHP acid in urine

The levels of HHP acid and MHHP acid in urine **(Paper II)** were determined as described elsewhere (Jönsson and Lindh 1996). Briefly, the acids were worked-up using reversed, solid phase extraction (SPE) and then derivatized using pentafluorobenzyl bromide (PFBBr) and analysed by GC-MS in the negative ion chemical ionisation (NICI) mode.

Determination of plasma protein adducts of HHPA and MHHPA

Internal standards

$^{2}H_{6}$-labelled HHP acid and $^{2}H_{8}$-labelled MHHP acid were synthesised as previously described by Jönsson and Lindh (1996).

External standards

Expired plasma from a blood bank were dialysed as described below. Solutions of HHP and MHHP acids were prepared by hydrolysis of the corresponding anhydrides in 0.01 M NaOH. Stock solutions of desired concentrations were then prepared by further dilutions in 0.01 M NaOH. Standards were prepared by addition of 100 µl aliquots of the stock solutions to a volume of dialysed plasma which corresponded to 1.0 ml of undialysed plasma.

Dialysis of plasma

One ml plasma was dialysed in dialysis tubing (SpectraPore; 2.0 ml/cm with a cut off at 12-14 kDa) against phosphate buffered saline (PBS) containing 2 mM EDTA, at 4°C over six days with stirring. The buffer was changed after 8 hours and then on days 2, 3 and 5.

Hydrolysis of adducts to HHP acid and MHHP acid

Dialysed plasma samples were mixed with 3 ml 0.1 M HCl and 100 µl of an internal standard solution containing 6 pmol each of $^{2}H_{6}$-labelled

HHP and $^{2}H_{8}$-labelled MHHP acids. The samples were incubated at 100°C for 2 hours and then allowed to cool to room temperature.

Extraction of HHP acid and MHHP acid

Varian C_{18} Bond Elut LRC columns (100 mg) connected to a Cerex SPE processor (Varian) operated at 20-60 kPa of nitrogen gas were conditioned by passing 5 ml of methanol followed by 2 ml of 0.1 M HCl. The hydrolysed plasma were loaded onto the columns, which trapped HHP and MHHP acids. The columns were washed with 2 ml of 10% methanol in 0.1 M HCl and then dried by the nitrogen gas flow for 1 min. The HHP acid was eluted first, into a set of test tubes using 1 ml 40% methanol in 0.1 M HCl. Thereafter the MHHP acid was eluted into another set of test tubes using 1 ml 60% methanol in 0.1 M HCl. The eluates were evaporated to dryness in a heating block at 70°C, in a nitrogen gas flow.

Derivatisation of HHP acid and MHHP acid

The dry residues were derivatised by addition of 250 µl of 0.1 M tetrabutylammonium hydrogen sulfate solution and 250 µl of 0.13 M pentafluorobenzyl bromide (PFBBr) solution. The samples were vortexed and placed in an ultrasonic bath for 90 min. When cool, 2 ml of hexane was added, the samples were vortexed and then centrifuged for 15 seconds at 1500*g* for phase separation. The test tubes were placed in a -20°C freezer until the aqueous phases were frozen. Thereafter, the organic phases were poured into a new set of test tubes and evaporated in a dry nitrogen gas flow at 50°C. The dry residues were dissolved in 150 µl of toluene and transferred to auto-sampler injection vials for analysis by GC-MS.

Analysis using GC-MS

The GC-MS equipment was a Carlo-Erba 8065 GC equipped with an A200S auto-sampler and a fused silica capillary column (30 m x 0.25 mm I.D.) with a DB-5 MS stationary phase which had a film thickness of 0.25 µm (J&W Scientific); connected to a VG Trio 1000 quadrupole MS (Fisons).

Samples (2 µl) were injected using a splitless injection technique with the injector temperature at 300°C. The split exit valve was kept closed for 0.5 min after the injection. For the analysis of di-pentafluorobenzyl hexahydrophthalate (PFB-HHP), the initial column temperature was 100°C for 1 min and then increased by 15°C/min to

320°C. For di-pentafluorobenzyl methylhexahydrophthalate (PFB-MHHP) the initial temperature was 110°C for 1 min and then increased by 8°C/min to 320°C. The MS interface was at 300°C and the ion source at 200°C. The column carrier gas was helium at a pressure of 150 kPa. The MS was in the NICI mode with ammonia as moderating gas. Selected ion monitoring (SIM) of PFB-HHP was performed at *m/z* 153 and 351 while *m/z* 159 and 357 were chosen for the internal standard. For PFB-MHHP *m/z* 167 and 365 were chosen and *m/z* 175 and 373 were used for the corresponding internal standard.

Quantification of adducts

Quantification was done using *m/z* 153 for PFB-HHP with *m/z* 159 for the internal standard and using *m/z* 365 for PFB-MHHP with its corresponding internal standard fragment at *m/z* 373. Simple linear regression was performed with the ratio between the external standard and the internal standard peak-area responses as the dependent variable in the plotting of the calibration graphs. The graphs were linear over the whole calibration range, but for quantifications of low concentrations a calibration graph in this region was normally used.

Determination of Hb adducts of HHPA and MHHPA

The levels of Hb adducts of HHPA and MHHPA were determined by Jönsson *et al*. (1996).

Fractionation of plasma proteins using ion exchange chromotography

Plasma (0.5-1 ml) were dialysed as described previously, for 5-6 days and then diluted 50:50 with 20 mM ethanolamine buffered at pH 9.5 (**Papers I and III**). Aliquots of sample (500 µl) were injected via a 1 ml loop into an ÄKTA purifier LC system (Pharmacia Biotech) fitted with a Mono Q HR 5/5 column. (Pharmacia Biotech). The equilibration buffer was 20 mM ethanolamine (pH 9.5). An elution buffer of 20 mM ethanolamine buffer with 1 M NaCl (pH 9.5) was used in a gradient from 0 to 20% in 5 column volumes (CV; 1 CV=0.98 ml) then to 40% in 20 CV and finally to 100% in 5 CV where it was kept for 4 CV. The mobile phase flow rate was 1.0 ml/min. One ml fractions were collected and agarose gel electrophoresis screens were run on the fractions (see below). In **Paper III**, the corresponding fractions from several ion exchange chromatography (IEC) runs were pooled, dialysed and analysed for HHPA adducts as described above. In addition, all fractions

containing SA from several runs were combined. The pooled sample was dialysed as described above and at the same time reduced in volume using polyethylene glycol (PEG) with a molecular weight of 20,000 in the dialysis buffer, (50 mg PEG/l PBS-EDTA buffer). The concentrated SA sample was analysed for SA content (determined by a double antibody RIA kit commercially available from Pharmacia) and SA adducts of HHPA (**Paper I**) and also further purified using gel filtration (**Paper III**).

Purification of SA using gel filtration

The SA sample from IEC was further concentrated by Amicon Centriprep and Centricon (**Paper III**). It was run on a screen and also analysed for levels of SA adduct of HHPA. Aliquots of the sample were injected via a 100 µl loop into the ÄKTA purifier LC system equipped with a Pharmacia Biotech Superdex 200 column (model 10/30). The mobile phase was 0.05 M NH_4HCO_3 with a flow rate of 0.7 ml/min. One ml fractions were collected and corresponding fractions from several runs were combined. These were analysed by agarose gel electrophoresis to identify proteins. The fractions were dialysed and concentrated in PBS-EDTA buffer with PEG (50 mg PEG/l PBS-EDTA buffer) to about 2 ml. These fractions were thereafter quantified for HHPA adduct content.

Gel electrophorosis

Agarose gel (0.8%) was prepared in barbital-sodiumbarbital buffer (0.075M, pH 8.6, 2mM EDTA) as described by Johansson (1972). Acrylamide gel (4-15%) in tris/glycin buffer (Biorad) was used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (**Paper III**).

Autoradiography

Plasma from the guinea-pig exposed to radioactive HHPA was dialysed against PBS-EDTA buffer for two weeks (**Paper III**). Two aliquots of the plasma containing 80 counts per minute (cpm) of activity were separated by SDS-PAGE. The gel was stained with Coomassie blue, destained and then treated with EN^{3}HANCE™ autoradiography enhancer (Dupont). It was then washed in 10% glycerol in water, wrapped in cellophane film, dried and exposed on CEA RP X-ray film between intensifying screens at -70°C for 15 months.

Synthesis of OAA-protein conjugates

Total plasma protein conjugate of HHPA

HHPA (61.5 mg) was dissolved in 0.5 ml dioxane and then slowly added to 10 ml human plasma at room temperature (**Papers I and III**). The pH was then adjusted to 8.0 using aqueous NaOH and the resulting solution was stirred overnight at 4°C. The conjugate was purified by dialysis (Spectrapor; cut off 3.5 kDa) against PBS buffer over 5 days and stored at -20°C.

HSA conjugate of HHPA and MHHPA

HHPA and MHHPA, respectively, were added to cooled solutions of HSA (37 mol/mol) in 0.1 M $NaHCO_3$. An Amicon ultra filtration cell (model 8200) was used for the purification of the conjugates. The proteins were lyophilized and reconstituted in 0.1 M $NaHCO_3$ before use (**Papers III and IV**).

Determination of specific antibodies

Specific IgE immunoblotting

Three inhibition mixtures were prepared by addition of either 3.0, 0.15 or 0 mg of HHPA- HSA conjugate to aliquots of 1 ml serum from two workers known to have HHPA specific IgE (**Paper III**). These mixtures were incubated at 4°C overnight, centrifuged and then diluted 1:40 with 5% dried milk in in tris buffer saline tween (TBST) buffer (10 mM tris, 150 mM NaCl 0.25% tween 20, pH 8.0). For each subject, three aliquots of HHPA-plasma protein conjugate were separated by SDS-PAGE. Non-conjugated plasma, as a control, was also separated adjacent to each of the 3 conjugate lanes. The proteins were blotted to a pre-conditioned Sequi-Blot PVDF membrane (Biorad) using a Semi-dry Electroblotter A (Ancos) at 150 mA for 3 hours. The transfer buffer contained 48 mM Tris, 39mM Glycin, 20% methanol and 1.3 mM SDS. The membrane was then blocked by incubation in 5% dried milk, made up in TBST buffer, for 2 hours at 21°C. The membrane was divided in three and incubated in the different inhibition solutions for 2 hours at 21°C. These were thereafter washed three times with 5% dried milk in TBST-buffer and incubated at 21°C for 2 hours with an ^{125}I-labelled rabbit/mouse monoclonal-antihuman IgE (Pharmacia) diluted to an activity of about 700000 cpm with 5% dried milk in TBST-buffer. After a final wash with TBST-buffer, the membranes were wrapped in cellophane and exposed on CEA RP X-ray film between intensifying screens at -70°C for 8 days.

Specific IgG immunoblotting

The HHPA-plasma protein conjugate was diluted 1:100 and 4 aliquots of 5 µl were separated by SDS-PAGE (**Paper III**). Non-conjugated plasma was also separated adjacent to each of the four conjugate lanes. The proteins were blotted and blocked with milk as described above for the specific IgE immunoblotting. The blocked membrane was cut in four, each with a conjugate and a non-conjugate plasma blot. Three of the blots were incubated for 2 hours at 21°C in serum (diluted 1:20 with 5% dried milk in TBST-buffer) from three different HHPA exposed workers known to have high levels of specific IgG. The fourth was stained with Coomassie-blue for identification of protein bands. The serum incubated membranes were washed three times with 5% dried milk in TBST-buffer and incubated at 21°C for 2 hours with an alkaline phosphatase conjugated rabbit-antihuman IgG (Dako) diluted 1:1700 with 5% dried milk in TBST-buffer. The membranes were then finally washed with TBST-buffer and developed using Nitro Blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

Analysis of specific IgE

Specific IgE antibodies were determined as previously reported by Welinder and Nielsen (1991). The synthesised OAA-HSA conjugates were bound to cyanogen bromide-activated filter paper disks for use in a radioallergosorbent test (RAST; Phadebas, Pharmacia) system (**Papers III and IV**). All samples were analysed in duplicates. The results were expressed as percentage specific binding (cpm of test disc minus cpm of HSA reference disc) of the total added radioactivity. The sample was classified as positive if the value was above that of the highest control subject.

Analysis of specific IgG

The specific IgG was analysed using an enzyme-linked immunosorbent assay (ELISA) as described by Welinder and Nielsen (1991). Polystyrene microtiter plates were coated with a solution of OAA-HSA conjugate and blocked for non-specific binding (**Papers III and IV**). Thereafter; (i) 100µl of a 1:50 diluted (PBS) solution of serum was added and incubated at 20°C for 60 min; (ii) 100 µl of an optimal dilution of alkaline phosphatase conjugated rabbit anti-human IgG (Dakopatts), was added and incubated at 20°C for 60 min; (iii) 100 µl substrate solution (disodium p-nitrophenol phosphate) was added and incubated at 20°C for 120 min. The results were read at 405 nm

(Titertek Multiscan). All samples were analysed in triplicates and results expressed as the absorbance values. A value exceeding the highest control subject was defined as positive.

Skin-prick test

To test for OAA sensitization, conjugates between HSA and HHPA and MHHPA, respectively, were prepared as described above (**Paper IV**). A solution of histamine chloride (10mg/ml) was used as a positive control and a 0.1 M $NaHCO_3$ solution as a negative control (Welinder et al. 1994). The reaction was classified positive if the diameter of the weal exceeded 2 mm and the negative control was not above 1 mm. For a standardised quantification of the skin-prick test (SPT) result, the mean of the widest diameter and that at 90° from the midpoint of this was assessed, and the ratio between this mean and the corresponding value of the histamine reaction was calculated and denoted as the SPT ratio.

Lung function test

Forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) were determined in accordance with the guidelines developed by the European Community for Coal and Steel (ECCS; Quanjer et al. 1993; Vitalograph S spirometer). The highest values ($FEV_1$and FVC) of three accepted curves were used. (**Papers III and IV**)

Medical examination

Extensive occupational and medical histories including smoking habits were obtained from self administered questionnaires and supplemented with interviews by a physician (**Papers III and IV**). Information about current and previous work tasks at the present workplace, WRS, such as symptoms from the eyes (lacrimation, itching, scratching, smarting and/or burning eyes); from the nose (blocked, runny, itchy and/or attacks of sneezing and/or bleeding); and from lower airways (dyspnoea, wheezing, chest tightness or dry cough) were collected. Symptoms were denoted WRS if they appeared in relation to occupational activities and improved during weekends and/or holidays. Atopy by history, defined as presence of asthma, hay fever, atopic eczema or urticaria during childhood or adolescence was also investigated.

Statistics

Bivariate associations between parametric values were assessed by linear regression or Spearman`s rank correlation (**Papers I-IV**). Trends

were evaluated by the Jonkheere-Terpsta test (**Paper IV**). The prevalence odds ratios (POR) with 95% confidence intervals (CI) was used for measuring the effects of exposure on immunological parameters and symptoms, respectively (**Paper IV**). As potential confounders age, gender, atopy and smoking were considered. For the WRS, specific IgE was also taken into the multivatiate model. Moreover, we tested whether these factors modified the effect of exposure. Statistically significant denotes $p< 0.05$ (two-tailed).

RESULTS WITH COMMENTS

Quantification of TPPA

Stability of adducts during storage

There was no degradation of TPPA of HHPA during a one year storage at -20°C (**Paper I**).

Dialysis

Dialysis was performed to eliminate free acids and other LMW chemicals that may interfere with the analysis. Thus, the optimal time for this step was evaluated (**Paper I**) For removal of free HHP acid in plasma, a dialysis time of one day was sufficient but for MHHP acid more than three days was required before the levels of free acid were below the detection limit. This observation may suggest that MHHP acid has a higher affinity for plasma proteins compared to HHP acid. A compromised dialysis time of six days was chosen to ensure complete removal of the free acids.

Hydrolysis

The protein bound anhydrides were released by hydrolysis. The optimum hydrolysis condition giving yields of the corresponding acids close to 100% was found to be with 0.1 M HCl for 2 hours at 100°C (**Paper I**). At these mild conditions there was no degradation evident, either of HHP acid or MHHP acid, as determined from normal plasma samples spiked with known amounts of the acids.

Solid phase extraction

HHP acid and MHHP acid were extracted from the hydrolysed samples using reversed SPE. The optimum extraction of the acids were obtained when HHP acid was eluted first, with 40% methanol in 0.1 M HCl, and MHHP acid was eluted directly after with 60% methanol in 0.1 M HCl (**Paper I**). Thus, the HHP and MHHP acids had to be analysed separately but the improvement in the analytical matrix for MHHP acid using this procedure justified this extra step.

Chromatography and mass spectrometry

PFBBr was used for the derivatisation of HHP and MHHP acids to PFB-HHP and PFB-MHHP, respectively. These derivatives show excellent properties both regarding chromatographic behaviour and sensitivity. For example, the instrumental detection limit for PFB-HHP was found

to be below 5 attomol (**Paper I**). The PFB-HHP was found to be separated relatively easily from other substances but a longer temperature gradient was required to separate PFB-MHHP from interfering compounds. For PFB-HHP, the fragment at *m/z* 153 was somewhat purer than *m/z* 351. For PFB-MHHP, both fragments, at *m/z* 167 and 365, were almost equally useful.

Detection limit

Detection limits were determined using plasma from ten personnel at our laboratory (**Paper I**). The plasma were dialysed as normally but to simulate the authentic samples which contain some free acids, 300 pmol each of HHP and MHHP acids were added to the first dialysis buffer. The detection limits were calculated as the concentrations corresponding to the peak-area ratios with the same retention time as PFB-HHP and PFB-MHHP plus three times the standard deviation of these values (**Table 2**). Because of their better detection limits, the fragments chosen for quantification were *m/z* 153 for HHPA and *m/z* 365 for MHHPA.

Table 2. Detection limits for HHPA and MHHPA.

Anhydride	Mass fragment (*m/z*)	Detection limit (fmol/ml plasma)
HHPA	153	60
HHPA	351	190
MHHPA	167	60
MHHPA	365	30

Precision

The between-day precisions were determined from duplicate analysis on different days of plasma samples from 16-24 occupationally exposed

workers. The values varied from 6-24% depending on the concentration of the adduct and the mass fragment used in quantification **(Paper I)**. Hence, despite the extremely low levels of adducts and the numerous steps in the procedure, it is still possible to quantify the levels of adducts with high precision. Deuterium labelled internal standards are a requirement for this high precision.

Evaluation of TPPA as biomarkers of exposure

Toxicokinetics of TPPA

The *in vivo* stability of the adducts were calculated in two workers ending employment. The elimination was found to follow a one compartment model with a half-time of 22 days for HHPA and 24 days for MHHPA, both in good agreement with the half-time of SA of about 20 days. In addition, the half-time was studied in 7 workers over a period of 28 to 35 days without exposure to HHPA and MHHPA (**Paper II** and **Table 3**). Most calculated values are in approximate agreement with the half-time of SA. However, one value is more than ten times higher than expected (worker 6) possibly due to an exposure of MHHPA during vacation.

Associations between air levels and TPPA

Workers exposed to time-weighted average air levels of HHPA between <1 and 340 µg/m^3 had plasma adduct levels between the detection limits of the method and 8.40 pmol/ml (**Paper I**). Exposures between 2 and 160 µg/m^3 of MHHPA resulted in plasma adduct levels between the detection limits and 19.0 pmol/ml. The Spearman`s rank correlation coefficient for HHPA was r_s=0.96 while that for MHHPA was r_s=0.86.

Correlations between single day urine samples and TPPA

The correlations between short-term biological monitoring using one day urine sampling and TPPA were good considering the fact that adducts show a mean integrated long-term exposure while the urine metabolites reflect only the exposure during one work shift. The Spearman`s rank correlation coefficient for HHPA was r_s=0.71 and that for MHHPA was r_s=0.81 (**Paper II**).

Table 3. *In vivo* half-time for HHPA and MHHPA adducts in workers during a leave of 28-35 days from work.

Worker[a]	Half-time for	
	HHPA adducts (days)	MHHPA adducts (days)
1	20	27
2	16	BDL[b]
3	22	BDL
4	20	17
5	35	BDL
6	ND[c]	287
7	27	27

[a]The order of the workers 1-5 is the same as for HHPA in **Paper II**. The results for worker 7 is new. Workers 1, 4, 6 and 7 are the same as those analysed for Hb adducts by Lindh and Jönsson (1998); plasma from the fifth worker in that study was missing.
[b]BDL=below detection limit
[c]The results were inconclusive when the plasma was finished.

Correlations between repetitively sampled urine and TPPA

Excellent correlations were seen between long-term exposure as assessed using repetitive urine sampling and anhydride plasma protein adduct levels. The linear regression correlation coefficient, r, was 0.97 for HHPA and 0.92 for MHHPA (**Paper II**). The slopes of the linear regressions indicate that at the same air exposure levels, MHHPA tends to give 3 times more adducts compared to HHPA.

Correlations between Hb adducts and TPPA

The correlations between Hb adducts and TPPA of HHPA were found to be high with the linear correlation, r=0.86 (**Paper II**).

Characterisation of plasma protein adducts

Adducts between HHPA and MHHPA and proteins in human plasma

Plasma from an HHPA exposed worker (**Paper III**) was separated using IEC. The eluted amount of HHPA-adducts in all collected fractions was 99% of the totally injected amount of adducts on the IEC column. The majority of the HHPAadducts, 88% of the totally eluted amount, was recovered in the fractions co-eluting with SA as identified by agarose gel electrophoresis. Further, fractions containing SA were pooled and fractionated using gel filtration. The eluted amount of HHPA adducts in all collected fractions was 108% of the total amount injected on the gel filtration column. The majority of the HHPA adducts, 98% of the totally eluted amount, was recovered in the fractions co-eluting with SA. These observations indicate that at least 85% of the adducts are bound to SA.

In **Paper I**, proteins in plasma from 10 HHPA and 10 MHHPA exposed workers were separated using IEC and the fractions containing the SA were analysed for adduct content. In addition, the TPPA levels and the concentration of SA were quantified. From these determinations it is possible to estimate the fraction of adducts bound to SA. Thus, for HHPA, 97 (range 60-120)% of the adducts were bound to SA. For MHHPA the corresponding fraction was only 64 (range 29-92)%.

Adducts between HHPA and proteins in guinea-pig plasma

Plasma from a guinea-pig exposed to HHPA was fractionated using IEC. The amount of HHPA protein adducts in all collected fractions was 84% of the totally injected amount of adducts. In the fractions co-eluting with SA, 96% of the totally eluted HHPA adducts was recovered. Further, the fractions containing SA were pooled and fractionated using gel filtration. The recovery of HHPA adducts from all fractions was 103% of the totally injected amount of adducts on the column. In the fractions co-eluting with SA, 93% of the totally eluted HHPA-protein adduct was recovered. It therefore seems that more than 75% of the HHPA had bound to SA.

Autoradiography of guinea-pig plasma
Plasma from a guinea-pig exposed to gaseous, radioactive HHPA was separated by SDS-PAGE and analysed by autoradiography. A single prominent band was seen at about 66 kD corresponding to the size of SA.

Comparison between TPPA and SA adducts
The quantification of exposure using TPPA is based on the amount of adducts per volume plasma. This could have a negative effect on the precision of the method as there is a natural variability in levels of SA in individuals. To study this possible error, plasma from ten HHPA and ten MHHPA exposed workers were analysed for SA adducts of HHPA and MHHPA and also TPPA of HHPA and MHHPA (**Paper I**). Although, the levels of SA in the subjects varied between 38-49 mg/ml, the linear correlations of TPPA of the anhydrides with SA adducts were very high (r= 0.94 for HHPA and 0.99 for MHHPA). Thus, the formation of adducts seems independent of the SA content of plasma at these exposure levels and the quantification of adducts as TPPA seems to be a fair indicator of adduct levels with SA.

Affinity of antibodies for plasma protein conjugates
Specific IgE
The affinity of IgE antibodies for different plasma protein conjugates of HHPA were investigated using an immunoblotting technique (**Paper III**). An *in vitro* synthesised HHPA-plasma protein conjugate and non-conjugated plasma (control) were run on SDS-PAGE gels, transferred to membranes and immunoblotted using sera from two HHPA exposed workers known to have HHPA specific IgE. The IgE in these sera had previously been inhibited to various extent using an HHPA-HSA conjugate. For both workers, there was one major band at about 66 kDa corresponding to the size of SA in all conjugate lanes, decreasing in intensity with increasing amount of inhibition of specific IgE in sera. There were also diffuse bands at about 28 kDa which could be inhibited by HHPA-HSA conjugate. There were no bands in the non-conjugated plasma lanes and no further bands appeared even after exposure on the film for a further month. This suggests that the specific IgE antibodies from exposed workers have a preferred affinity for the SA-conjugates in plasma.

Specific IgG

The affinity of IgG antibodies for different plasma protein conjugates of HHPA were also investigated using immunoblotting (**Paper III**). The synthesised HHPA-plasma protein conjugate and non-conjugated plasma were separated on a SDS-PAGE gel and transferred to PVDF membrane. The immunoblotting was performed using sera from three HHPA exposed workers. A dominant band was seen at 66 kDa in the HHPA-conjugate lane for all three workers. The corresponding bands in the non-conjugated plasma lanes were very weak. There were diffuse bands at about 50 kDa in the HHPA-conjugate lanes which were also present in the non-conjugated plasma, probably corresponding to IgG. There were weak bands at about 28 and 80 kDa in the HHPA-conjugate lanes which were absent in the non-conjugated plasma lanes. Some smaller proteins seen in the Coomassie Blue stained gel, (e.g. at 25 kDa) were absent in the blottings. Thus, the specific IgG seem to have a preferred affinity for the SA-conjugates in plasma.

Exposure-response relationships

Correlations between TPPA and specific antibodies

In the group of 139 HHPA and MHHPA exposed workers, 21% were found to be positive for specific IgE and 20% for IgG (**Paper IV**). Low but significant correlations were seen between TPPA of HHPA, and specific IgE and IgG, respectively using Spearman`s rank correlation test. When the workers were divided into four similar sized groups according to their adduct levels, significant exposure-response relationships were observed between HHPA adducts and, specific IgE and IgG, respectively and between MHHPA adducts and specific IgG (logistic regression and trend test). There were only small changes in the estimates of the PORs when age, gender, atopy and smoking was taken into the multivariate model.

Correlations between TPPA and work related symptoms

Considering WRS for the 139 exposed workers, 27% had symptoms from the nose, 21% had symptoms from the eyes, 11% reported symptoms from the lower airways and 8% had nose bleed (**Paper IV**). On dividing the workers into four similar sized groups a significant exposure-response relationship between HHPA exposure and symptoms from the eyes was found. The subjects in the highest HHPA exposed group tended to have more frequent symptoms from the nose as compared with the lowest exposure group (POR 2.5, CI 0.8-7.7). When

gender was taken into the multivariate model the POR increased to 3.4 (CI 1.0-11.0). On the other hand, when specific IgE was taken into the multivariate model for HHPA exposure the PORs for eyes and nose symptoms and nose bleed decreased in all exposure groups. However, for symptoms from the eyes there was still an exposure-response relationship, with a POR of 4.0 (CI 1.0-16.4) for the highest exposure group, and for nose symptoms there was also a tendency to such a relationship while that for nose bleed was completely abolished. For MHHPA exposure there were no obvious exposure-response relationships seen with WRS and there were only small changes in the estimates of PORs when age, gender, atopy, smoking and specific IgE were taken into the multivariate model.

GENERAL DISCUSSION

Biological monitoring using adducts between endogenous proteins and xenobiotics as biomarkers of long-term exposure was an idea first suggested by Ehrenberg and co-workers in 1974. After more than 25 years, despite the potential the adducts possess as biomarkers, their use for exposure assessments is still very limited. One reason might be that the work-up procedures for quantification of protein adducts tend to be laborious and tedious. In this work we have developed a method to quantify the levels of TPPA which is easy and convenient; dispensing with the tedious, labour-intensive protein isolation steps. This achievement allowed us to analyse plasma from as many as 141 exposed workers.

There are several reports indicating that HHPA and MHHPA are sensitising at very low µg/m^3 exposure levels (Welinder 1991, Nielsen 1992, Welinder *et al*. 1994 and 2001, Nielsen *et al*., in press). Thus, to be able to monitor such low exposures using protein adducts, it was necessary to develop a method that is highly sensitive but also has a very clean analytical matrix. Derivatisation of carboxylic acids with PFBBr was first used by Ehrsson (1971) and this regent has previously been described to yield highly sensitive derivatives of HHP and MHHP acids. (Jönsson and Lindh 1996**,** Lindh and Jönsson 1998a). In addition, the pentafluorobenzyl esters of HHP and MHHP acids show very good chromatographic properties.

In order to achieve a clean analytical matrix, the work-up procedure was carried out in a laboratory where neither HHPA nor MHHPA was handled. LMW contaminants as well as free HHP and MHHP acids were removed by extensive dialysis. Also, mild hydrolysis conditions were used to release the bound anhydrides from the proteins. Thus, a two-hour hydrolysis with dilute hydrochloric acid was sufficient to release the adducts without breaking additional chemical bonds in the proteins to any significant extent. Using chemicals of the highest purity, when available, also contributed towards a cleaner analytical matrix. Even after taking all these precautions, it was still necessary to elute the HHP and MHHP acids separately with different methanol concentrations in the SPE and also to use a longer chromatographic separation for the derivative of MHHP acid.

For estimation of the detection limit, blood was sampled from 10 personnel at our laboratory. Thus, we cannot be certain that they were totally unexposed to HHPA or MHHPA; three of the samples were

higher for HHPA than the rest, on both monitored mass fragments. Excluding these samples would have given a detection limit for HHPA below 30 fmol/ml. Also, the detection limits for the TPPA methods were calculated as the amounts corresponding to the mean of the peak area ratios of the samples with the same retention time as PFB-HHP and PFB-MHHP, plus three times the standard deviation of these ratios. This definition tends to give comparatively high estimates of the detection limit. Furthermore, all ten analysed samples had peaks at the retention time for PFB-HHP and therefore contain useful information on exposure even if the results were below the reported detection limits of the method. These arguments justify the quantification and use of adduct levels which were below the detection limits.

The evaluation of the hydrolysis conditions showed that almost all bound anhydride was released from the protein. Furthermore, the precision of the method was found to be sufficiently high. Thus, the developed method for analysis of TPPA is accurate and also reliable.

There were high correlations between estimates of single day exposures using air levels or urine metabolites, with TPPA of HHPA and MHHPA. Furthermore, the correlations with the adducts were even higher with long-term exposures as assessed through repetitive urine sampling over one month or through Hb-adducts. It is possible to monitor extremely low but still clinically relevant exposure levels with the method. The adducts seemed to be stable *in vivo* over the whole life-time of SA. Thus, TPPA of HHPA and MHHPA are excellent biomarkers of long-term exposure.

In many of the symptomatic workers anhydride specific IgE antibodies were found. Thus, a Type-1 allergy mechanism seems reasonable in those cases. The relevance of the OAA specific IgE antibodies for induction of clinical symptoms has been proved by Nielsen *et al*. (1994). The binding of the anhydrides to endogenous proteins is an important step in this IgE mediated mechanism. In this study we found that HHPA and MHHPA mainly bound to SA in plasma. It has been shown that intradermal injection of *in vitro* synthesised conjugates between rat SA and anhydrides induce a production of anhydride specific antibodies in rats (Zhang *et al*. 1998). Thus, SA adducts of the anhydrides are immunologically active. However, the amount of SA conjugates used in that study were very high. Our study found that in exposed workers, less than one out of one million SA was conjugated with anhydride at exposure levels sufficient to cause sensitisation in a large fraction of the these workers. Lindh *et al*. (1999)

found an extensive labelling of proteins in the upper airways following exposure of rats and guinea-pigs to gaseous, radioactive HHPA. Thus, it is possible that these heavily labelled proteins are responsible for the immunological response. Conjugates of hexamethylene diisocyanate with proteins from the human lung epithelial cell lines have shown immunological activity (Wisnewski *et al*. 1999).

There were significant exposure-response relationships between TPPA of HHPA and specific IgE antibodies. In another study on the same workers as studied in this thesis when air levels and urinary metabolites were used for the exposure assessments, the correlations with specific IgE were not so strong (Nielsen *et al*., in press). In addition, the exposure levels giving rise to sensitisation in the study by Nielsen *et al*. seemed to be higher compared to in the present study. Thus, in this group of workers the use of TPPA provided information on exposure-response relationships which was not feasible by the other methods for measurements of exposure. On the other hand, in a recent study by Welinder *et al*. (2001) on another group of workers, similar results to ours were found, both regarding the degree of correlations and the exposure levels giving rise to antibodies. The study by Welinder *et al* was a prospective study which can estimate the exposure better compared to the present cross-sectional study since the exposures in the former study were followed during the whole exposure period. However, Welinder and co-workers used air monitoring for exposure assessments and although air measurements were taken many times, overall they represented only a very small fraction of the total exposure time.

Exposure-response relationships were also found between the TPPA of HHPA and WRS. The associations found by Nielsen *et al*. (in press), both for exposure assessment using air sampling and also using metabolites in a single urine sample, seem to be rather similar to those found in this work. Thus, while specific IgE seem to be related to an extended, long-term exposure, WRS seem to be more closely associated with a more recent exposure.

MHHPA seems to give about three times as much adducts as HHPA at the same external exposure levels and, thus, it would be interesting to see whether MHHPA is more allergenic than HHPA. However, while TPPA of HHPA were highly correlated with specific IgE and IgG and WRS, TPPA of MHHPA were significantly associated only with the specific IgG. This result is further supported in the study by Nielsen *et al*. (in press) where a correlation was seen between HHP

acid in urine and specific IgG antibodies while no such relationship was found between MHHP acid in urine and the specific IgG. Thus, there are reasons to believe that HHPA may be more sensitising than MHHPA. There are, however, some alternative explanations for this observation. For example, there is strong cross-reactivity between HHPA and MHHPA antibodies and exposure to HHPA could therefore mask the IgE-effect from MHHPA. A look at the workers positive for specific IgE in the lowest MHHPA-exposure group identifies those workers as mainly HHPA exposed. But this still does not explain the lack of associations in all four exposure groups between MHHPA exposure and WRS. An alternative 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 sensitisation. There might be some support for this. For HHPA the threshold for induction of IgE seems to be lower than that of IgG which is in agreement with the data from Welinder *et al.* (2001). Also, the induction of IgG for MHHPA seems to start at a lower level than for HHPA. Thus, the threshold for IgE response for MHHPA may well be below the monitored exposure levels of this investigation.

The estimates of the PORs for WRS from exposure to HHPA decreased when specific IgE antibodies were taken into the multivariate model which is expected for a type-1 allergen. However, a significant exposure-response relationship was still evident for symptoms from the eyes. There was also a tendency to an exposure-response relationship for nose symptoms. Thus, the presence of specific IgE does not explain all WRS found in this study. Co-exposure to other chemicals might be an explanation for the workers without specific IgE but with symptoms. Exposure to DMBA has been reported to cause a dose dependent increase in the metachromatic cells and eosinophils in the nasal mucosa of non-atopic male volunteers (Irander *et al.* 1997). Another possible explanation could be that there are antibodies but these are directed against other carrier proteins than the SA used in the analytical tests. In this study it was shown that the antibodies mainly had affinity for the SA in an HHPA-conjugate mixture of different plasma proteins. Thus, from this point of view, SA seem to be a good choice of carrier protein for RAST and ELISA. However, the number of workers examined was small and it also remains to study conjugates of proteins other than those in plasma for antibody affinity. On the other hand, it seems reasonable to conclude that there must be other, yet unknown mechanisms behind airways diseases induced by OAA and other LMW chemicals. This is

exemplified by the isocyanates whose pathomechanisms behind adverse effects are seen to be much more complicated and obscure than OAAs. Specific IgE for these chemicals is seldom found in exposed, symptomatic workers (Baur *et al*. 1994).

In studies of natural protein allergens, air levels in the low ng/m^3 region seem to induce the production of specific IgE, for example rat urinary protein, α-amylase or latex (Baur *et al*. 1998, Nieuwenhuijsen *et al*. 1999, Heederik *et al*. 1999). In this study of two LMW chemicals, the levels giving sensitisation were three orders of magnitude higher. However, it has been reported that the majority of inhaled OAAs are hydrolysed to the corresponding acids which are rapidly excreted through urine and that probably less than one percent binds to proteins (Jönsson and Skerfving 1993). Thus, these LMW chemicals seem to be nearly as potent allergens as naturally occurring proteins.

Hoet and Haufroid (1997) have defined criteria for an ideal biomarker. Thus, a biomarker should (i) specifically assess the exposure to the chemical under investigation, (ii) be sufficiently sensitive to detect low exposure levels, (iii) vary quantitatively with the exposure levels and the risk of adverse effects, (iv) give more information on potential health risk compared to air monitoring, (v) be stable enough to allow storage, (vi) be non-invasive and ethically acceptable, (vii) be possible to quantify by an analytical method with sufficient accuracy, specificity and sensitivity, (viii) not need a too time consuming, complex or expensive method.

TPPA of HHPA and MHHPA fulfil most of the criteria required for a good biomarker. These adducts are formed only from exposure to HHPA or MHHPA**,** respectively, and they are analysed with a highly selective technique, GC-MS. Thus, the TPPA of HHPA and MHHPA can indeed be considered to be specific biomarkers. It is possible to determine very low exposures using TPPA of the anhydrides. However, to obtain exposure response relationships for MHHPA, quantification of even lower exposure levels would probably be necessary. There were very high correlations between the mean urinary metabolite levels over a one month period and the adduct levels. In addition, application of the TPPA as biomarkers in a group of HHPA and MHHPA exposed workers gave significant exposure-response relationships with regard to both specific antibodies and WRS. The TPPA therefore seemed to vary quantitatively with both exposure and risk. Collection of personal air samples are laborious and it is often not possible to monitor exposures during more than a few days. TPPA show integrated exposures over a

period of more than a month. This makes the exposure assessments using TPPA more robust compared to air sampling. Furthermore, it is possible to monitor retrospective exposures with TPPA. Also, the adverse effects of anhydrides are likely to be related more to internal exposure than external. Thus, TPPA give more information on potential health risk compared to air monitoring. The TPPA of HHPA and MHHPA are stable at storage. There are very little risks connected with collection of blood samples.

Since TPPA have been found to quite successfully fulfil the criteria above for good biomarkers, it follows that the analytical quantification method must be considered to be sufficiently accurate, specific and sensitive. Furthermore, the method is relatively practical and simple. Mass spectrometry has been considered to be an expensive technique but has lately become more accessible for routine analysis.

Studies on exposure-response relationships are necessary in risk estimations on which OELs can be based. These limits are in turn important in health surveillance and prevention of diseases. However, there are currently no OELs set for HHPA and MHHPA. Earlier studies on exposure-response relationships of HHPA and MHHPA suggest an OEL of less than 10-20 $\mu g/m^3$ (Nielsen *et al*., in press). This was an air level that was seen to prevent symptoms in IgE sensitised workers but the limit was insufficient for prevention of sensitisation. However, the results from our study as well as data from Welinder *et al*. (2001) suggest this value to be even lower. We found evident exposure-response relationships with both symptoms and with specific IgE down to 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 from the eyes and nose was 9 and 17%, respectively. These values could have been influenced by co-exposure to MHHPA and the presence of background levels of symptoms in the normal population; the corresponding levels of WRS in the control group were 14 and 16% from the eyes and nose, respectively (Nielsen *et al*., in press). 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. Thus, based on our findings and with support from the results of Welinder *et al*. (2001) and Nielsen *et al*. (in press) we would suggest a biological OEL of 60 fmol/ml plasma for TPPA of HHPA. Further studies need to be conducted to establish a clearer

exposure-response relationship for MHHPA. Meanwhile, a similar OEL to that suggested for HHPA may be applicable also for MHHPA.

CONCLUSIONS

A method for quantification of TPPA of HHPA and MHHPA was developed. The method is relatively quick and simple. It is accurate and has a high precision and a very low detection limit.

The elimination half-time of TPPA of HHPA and MHHPA was found to correspond well with the half-time of SA in plasma. The adducts therefore seem to be stable *in vivo.*

There were high correlations between short-term exposure measures and adduct levels. The correlations were even higher when the mean levels of HHP and MHHP acid in urine sampled over four weeks were correlated with TPPA levels at the end of that period. There was also a high correlation between Hb adducts of HHPA and TPPA of HHPA. It is possible to monitor very low exposures that are relevant at the workplaces. The TPPA are specific to HHPA and MHHPA exposures and have several advantages compared to air monitoring. The adducts are stable during storage and the collection of blood samples is ethically acceptable. Thus, TPPA can be considered to be excellent biomarkers of long-term exposure.

SA was identified as the major protein in plasma which forms adducts with HHPA and MHHPA. Minor bindings to other proteins were also observed.

HHPA-specific IgE and IgG in serum of exposed workers showed a preferred affinity for SA in an *in vitro* synthesised HHPA-plasma protein conjugate. However, the antibodies showed an affinity also for some other protein conjugates.

There were significant exposure-response relationships for HHPA with specific IgE and IgG antibodies and WRS down to adduct levels of 40 fmol/ml plasma. The exposure-response relationships for MHHPA was less obvious with a significant association only with specific IgG. Thus, TPPA can also be considered to be appropriate biomarkers of risk.

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ERRATA

Paper I

Page 158, Paragraph 2, Chromatography and mass spectrometry: Line 14, “5 mol (injected ammount)” should read “5 attomol (injected amount)”.

Page 159, Fig 5; Labelling of axis in Fig.5 (a) is missing. It should be “HHPA-adducts/fmol ml^{-1} plasma” on the x-axis and “HHPA-adducts/fmol mg^{-1} albumin” on the y-axis.

Paper II

Page 135, Paragraph begining “Correlations between total plasma protein adducts and urinary metabolite levels; line 8, “830 fmol/ml” should read “770 fmol/ml”.

Paper III

Page 1021, Summary; line 18 (results) “for GPs>74%” should read “for GPs>75%”.