

Is it possible to improve the patch-test diagnostics for isocyanates? A stability study of petrolatum preparations of diphenylmethane-4,4'-diisocyanate and polymeric diphenylmethane diisocyanate

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We have previously shown that the concentration of diphenylmethane-4,4'-diisocyanate (4,4'-MDI) in commercial test preparations was so low that patch testing with the same was not reliable. The stability of 4,4'-MDI in petrolatum (pet.) was compared with pet. preparations of polymeric diphenylmethane diisocyanate (PMDI), which consists of a complex mixture of monomeric isomers and oligomers of MDI. Preparations of 4,4'-MDI and PMDI were stored under 3 different conditions, i.e. at room temperature, refrigerated and frozen. They were analysed continuously during 1 year with regard to the content of 4,4'-MDI, 3-ring oligomers and 4-ring oligomers using liquid chromatography–mass spectrometry. PMDI preparations kept frozen were stable for a year. All other preparations failed to fulfil the requirements of stability, i.e. $\pm 20\%$ of the initial concentration. Storage in a freezer prolonged the lifetime for 4,4'-MDI. The decrease in concentration for preparations kept at room temperature and refrigerated was less rapid in PMDI preparations than in 4,4'-MDI preparations. PMDI preparations are better suited for patch testing patients exposed to MDI because they are more stable and homogeneous than 4,4'-MDI preparations. They better reflect possible allergens that workers are exposed to because products used in industry contain both monomers and oligomers.

Key words: allergic contact dermatitis; CAS 101-68-8; CAS 9016-87-9; diphenylmethane-4,4'-diisocyanate; MDI; patch-test preparation; PMDI; polymeric MDI; polyurethane; stability.
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Accepted for publication 13 September 2006

Introduction

Isocyanates, compounds containing the functional group --N=C=O , are frequently used in industry in the production of polyurethane (PUR) products such as soft and rigid foam, elastomers, lacquers, adhesives and coatings. Isocyanates can be classified based on the number of --N=C=O groups in the molecule (i.e. monoisocyanates contain one --N=C=O group, diisocyanates contain two --N=C=O groups and polyisocyanates contain multiple --N=C=O groups).

In the year 2000, more than 4 million tons of isocyanates were produced worldwide (1). In Europe alone, there are about 1000 employees in 9 plants that manufacture isocyanates and around 72 000 employees in 6000 facilities producing PUR (2). In spite of the numerous amount of

workers exposed and the fact that isocyanates theoretically are potent contact sensitizers, reports on allergic contact dermatitis from isocyanates are few in number. It has been suggested that the strict rules applied to isocyanate-associated work to avoid respiratory problems have contributed to minimizing contamination of the skin, and thus also contact allergy to isocyanates (3). However, reports where patients to a higher extent reacted to their own isocyanates-based work products, but not to any commercially available patch-test preparation of isocyanates caused us to consider another explanation, viz that the quality of these preparations was insufficient (4–7). Therefore, we performed chemical analyses on commercially available patch-test preparations of 4 diisocyanates used for detection of contact allergy to

isocyanates. The preparations were obtained from 13 dermatology departments and from 2 major European suppliers of patch-test allergens, and they consisted of diphenylmethane-4,4'-diisocyanate (4,4'-MDI), 2,4-toluene diisocyanate (2,4-TDI), 1,6-hexamethylene diisocyanate (1,6-HDI) and isophorone diisocyanate (IPDI) (7, 8). It was shown that the patch-test preparations of 2,4-TDI, 1,6-HDI and IPDI were fairly stable (7), but that the concentration of 4,4'-MDI in commercial test preparations was so low that patch testing with the same could not be considered reliable (8).

The present study investigates the stability over time of petrolatum (pet.) preparations of 4,4'-MDI when stored under different conditions. The stability of 4,4'-MDI in pet. is also compared with that of pet. preparations of a product called Pedilen, which consists of polymeric MDI (PMDI). PMDI is the technical grade of MDI used in industry and its composition varies with the manufacturer. However, PMDI products usually consist of a complex mixture of 25% to 80% 4,4'-MDI and oligomers (i.e. polyisocyanates) containing an increasing number of rings and

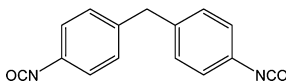
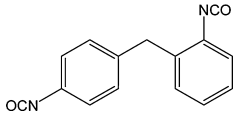
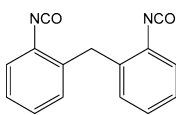
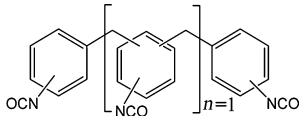
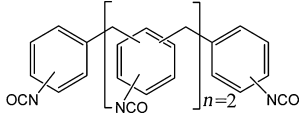
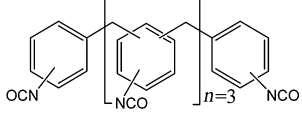
other minor isomers such as 2,4'-MDI and 2,2'-MDI (Table 1) (9). Pedilen came to our knowledge during an investigation of products used in the manufacture of prosthetics at a department of orthopaedic. When planning this study, we reviewed various PMDI products with regard to their composition, and we decided to use Pedilen because its composition (Table 1) corresponds well with the general composition of the most frequently used PMDI products in industry and as it was already in our possession.

Materials and Methods

Chemicals

Toluene, isooctane and methylene chloride were obtained from Lab-Scan (Dublin, Ireland), methanol and ethanol from Kemetyl (Stockholm, Sweden), di-*n*-butylamine (DBA) from Aldrich Chemie (Steinheim, Germany), *d*₉-labelled di-*n*-butylamine (DBA-*d*₉) from Synthelec (Lund, Sweden), 4,4'-MDI from TCI Europe N.V. (Zwijndrecht, Belgium), 617P21-Hardener for 617H37 PEDILEN (Pedilen) from Otto Bock Orthopedic Industry of

Table 1. Composition of the PMDI product Pedilen when quantified using a Chemiluminiscent nitrogen detector

Isocyanate	Formula	Molecular weight (g/mole)	Content in Pedilen (%)
4,4'-MDI		250	38.9
2,4'-MDI		250	0.6
2,2'-MDI		250	Not detected
3-ring oligomers		381	24.6
4-ring oligomers		512	8.6
5-ring oligomers		643	3.9
Not quantified compounds			23.4

PMDI, polymeric diphenylmethane diisocyanate; MDI, diphenylmethane diisocyanate.

Canada Ltd (Ontario, Canada), caffeine from Sigma Chemical (St Louis, MO, USA).

Calibration standard solutions and internal standards

Samples were prepared by accurately weighing about 15 mg of Pedilen into each of 2 test tubes. 10.0 ml toluene was added into each test-tube, and to 1 tube 50 μ l DBA was added and to the other 50 μ l DBA- d_9 . The test-tubes were shaken and placed in an ultrasonic bath for 10 min. The solvent and excess reagent were removed by evaporation of the samples, and the dry residues were dissolved in 10.0 ml methanol, respectively. The test-tubes were again shaken and placed in an ultrasonic bath for 10 min. The solutions were further diluted in methanol to appropriate concentrations for characterization using liquid chromatography–mass spectrometry (LC-MS) (Fig. 1) and quantification using a chemiluminescent nitrogen detector (CLND) (Table 1). Stock solutions of the calibration standard in the concentration 10 μ g/ml with respect to monomeric 4,4'-MDI-DBA derivative and of the internal standard (IS) in the concentration 10 μ g/ml with

respect to monomeric 4,4'-MDI-DBA- d_9 derivative were prepared by dilution in methanol.

Apparatus

Liquid chromatography–mass spectrometry. Analyses of the 4,4'-MDI and 3- and 4-ring oligomer content in the patch-test preparations were performed using a Quattro LC quadrupole mass spectrometer (Waters, Altrincham, Cheshire, UK) in the electrospray mode monitoring positive ions. The capillary voltage was 4.0 kV, the temperature of the ion source was 130°C and the desolvation temperature was 200°C. Quantifications were made by selected ion recording monitoring the molecular ions (Fig. 1). Quantitative measurements were made by calculating the response ratio between the analyte (i.e. as a DBA derivative) and the deuterium-labelled IS.

The mass spectrometry instrument was connected to Shimadzu LC10 ADvp LC-pumps (Shimadzu, Kyoto, Japan). 20- μ l loop injections containing a 4- μ l sample plug surrounded by 16 μ l of water were made using a CTC-Pal LC autosampler (CTC, Zwingen, Switzerland). The monomeric and oligomeric MDI-DBA and MDI-DBA- d_9 derivatives were analysed using mobile phase A with a composition of water/acetonitrile/formic acid of 95/5/0.05 (v/v/v) and mobile phase B with a composition of 5/95/0.05 (v/v/v) and with a flow rate of 70 μ l/min. Analysis started with 15-min gradient elution from 50% B to 65% B, then gradient elution to 100% B for the next 5 min and finally isocratic elution at 100% B for 10 min. The liquid chromatography column used was an Xterra[®] C18, 50 $\times$ 1.0 mm with 2.5 μ m particles from Waters (Milford, MA, USA).

Chemiluminescent nitrogen detector. The concentrations of each isocyanate derivative used in the calibration standard and IS were determined using LC-CLND. The Antek 8060 CLND is a nitrogen-specific detector where the response from the detector is proportional to the amount of nitrogen in the sample. Caffeine was used as external standard. On the Antek 8060 CLND, the pyrolysis temperature and the temperature of the membrane dryer were set to 1050 and 60 °C, respectively. The inlet oxygen and argon flows were 130 and 170 ml/min, respectively. The pressure in the reaction chamber was 20 torr, and the ozone flow in the reaction chamber was 25 ml/min. The photomultiplier voltage was set to 750 V. The CLND was connected to a Phoenix 40 micro LC-pump (Carlo Enba, Milan, Italy). Elution was performed by using a mobile phase A with a composition of methanol/water 5/95 (v/v) and mobile phase B with a composition of methanol/water

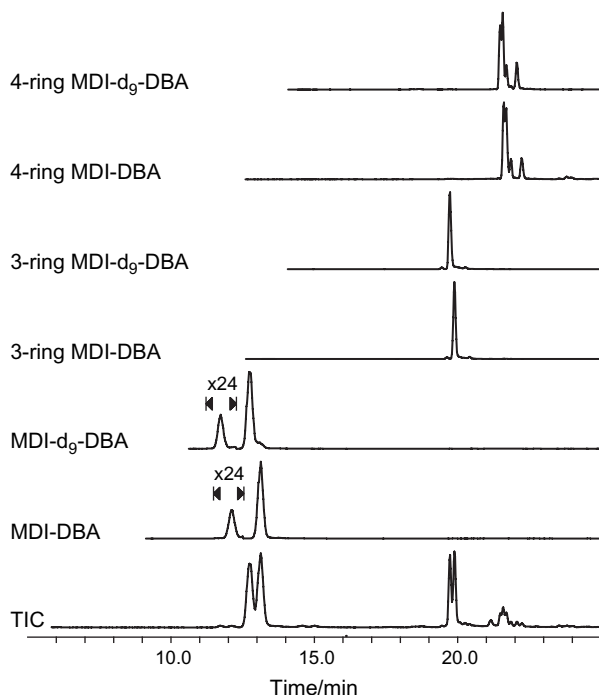


Fig. 1. Liquid chromatography–mass spectrometry chromatograms of monitored di-*n*-butylamine (DBA) and DBA- d_9 derivatives in a Pedilen-sample treated according to the work-up procedure. Chromatograms of diphenylmethane diisocyanate (MDI)-DBA and MDI-DBA- d_9 show peaks of 2 monomeric isomers, viz. the molecular ions of 2,4'-MDI-DBA (enlarged 24 $\times$) and 4,4'-MDI-DBA as well as 2,4'-MDI-DBA- d_9 (enlarged 24 $\times$) and 4,4'-MDI-DBA- d_9 .

95/5 (v/v) and with a flow rate of 100 µl/min. The analysis of PMDI started with 10 min of isocratic elution at 70% B, then gradient elution to 100% B during the next 40 min and finally isocratic elution at 100% B for 10 min.

General course and design of investigation

Preparation of 4,4'-MDI and Pedilen in pet. for storage. All pet. patch-test preparations of 4,4'-MDI and Pedilen were prepared under nitrogen in a transparent box specially developed to minimize any contact between the isocyanates and air during the preparation. The solid flakes of 4,4'-MDI were ground in the box and thereafter about 1 g of the ground 4,4'-MDI was weighed into a plastic jar. Pet. was added so that the concentration of 4,4'-MDI in the pet. preparation was about 2%. The 4,4'-MDI was evenly dispersed in the pet. by careful stirring between 5 and 10 min. A preparation containing Pedilen of about 2% pet. was prepared in the same manner, but since Pedilen is a viscous liquid no grinding was needed. Each preparation was dispersed into 9 disposable plastic syringes equipped with a plastic stopper.

The 9 preparations of each investigated substance (i.e. 4,4'-MDI and Pedilen) were stored for 1 year in 3 different places, viz 3 syringes were stored at room temperature (22°C), 3 in a refrigerator (8°C) and 3 in a freezer (–18°C).

Withdrawal of samples. Triple samples from all 18 syringes were worked up immediately after the preparation and analysed to get a start value of the concentrations. During the 1st month, triple samples of each syringe were withdrawn and analysed once a week. During the 2nd month, triple samples were withdrawn and analysed every other week. Thereafter, the withdrawal and analysis of samples occurred with longer and longer time intervals, viz at first a month apart and thereafter 2 months, 3 months and finally 4 months apart.

Work-up procedure. Triple samples were made from each syringe in the following way: about 100 mg of the pet. preparation was accurately weighed into a test-tube. The isocyanate groups in the pet. preparation were reacted with DBA in excess by adding 5.0 ml of 0.050 M DBA in methylene chloride to the test-tube (Fig. 2), and the sample was treated in an ultrasonic bath for 30 min. Thereafter, 5.0 ml of toluene was added followed by treatment in the ultrasonic bath for another 20 min.

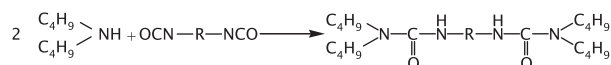


Fig. 2. General reaction describing the conversion of reactive diisocyanate into a stable urea derivative by reacting the diisocyanate with the secondary amine, dibutylamine.

The sample was diluted 100× in toluene (at the end of the study preparations stored in room temperature were only diluted 10× because the concentration of isocyanate in these samples was low). 1.0 ml was transferred into a new test-tube and 100 µl IS (10 µg/ml with respect to 4,4'-MDI-DBA-d₉) was added. The sample was evaporated to dryness in a vacuum centrifuge, and extraction was performed by adding 5.0 ml isooctane and 5.0 ml methanol/water (9/1; v/v) and by shaking vigorously for 10 min. The isooctane phase was discarded and the methanolic phase was washed with 5.0 ml isooctane, and then it was evaporated to dryness. The residue was dissolved in 0.5 ml methanol for LC-MS analysis. The main aim was to analyse the 4,4'-MDI content in PMDI and compare it with the content of the preparations containing pure 4,4'-MDI. However, the content of 3- and 4-ring oligomers in Pedilen was also analysed.

A calibration series was made by spiking aliquots of 1.0 ml toluene with the stock solution of the calibration standard containing DBA-reacted Pedilen to 7 concentrations in the range of 0–3.0 µg/ml with respect to 4,4'-MDI, after which they were treated according to the work-up procedure and analysed. Half way through the study, the calibration curve for the preparations based on PMDI was lowered to the range of 0–1.5 µg/ml, because the concentration of PMDI in these preparations had decreased.

The quantification limit was 0.04% (w/w), 0.025% (w/w) and 0.01% (w/w) for 4,4'-MDI, 3-ring oligomer and 4-ring oligomer, respectively.

Criterion for stability. Isaksson et al. stated in 2000 that there is a lack of a universal agreement with respect to the residual amount of active ingredient in a test preparation that has to be present to consider the preparation stable. However, they reported that according to personal communications with the manufacturers, the prerequisite of the content of preparations in the TRUE Test™ (Mekos, Hillerød, Denmark) and patch test allergens from Hermal (Reinbek, Germany) was ±20% of the concentration given on the label (10). Therefore, we also chose this limit.

Repeatability. To determine the repeatability of the method, a sample from the determination of the start concentration in the preparations containing PMDI was used. Aliquots of 100 µl of the stock solution of this sample, i.e. a solution where the pet. preparation had been diluted in 5.0 ml of 0.010 M DBA in methylene chloride and thereafter 5.0 ml of toluene, were transferred to 10 test-tubes and diluted in 900 µl of toluene, treated according to the work-up procedure and analysed. The repeatability was expressed as the coefficient

of variation (C.V.), i.e. the standard deviation expressed as a per cent of the mean.

Statistical calculations. To compare the variation of the concentration of 4,4'-MDI between triple samples of preparations containing pure 4,4'-MDI and preparations containing PMDI statistical analyses were carried out using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). The variation was analysed by comparing the C.V.s of triple samples of preparations containing 4,4'-MDI with the C.V.s (with regard to the 4,4'-MDI content) of triple samples of preparations containing PMDI. The Mann–Whitney test was used because the number of observations was small.

Results

The composition of the PMDI product Pedilen, which was used in the study, is shown in Table 1. The 23.4% that is unaccounted for, probably, derives from higher oligomers not detected with the used method (11). The stability, with respect to the 4,4'-MDI content, of the preparations containing pure 4,4'-MDI and the preparations containing PMDI when stored in a freezer, a refrigerator and at room temperature, respectively is shown in Figs 3 and 4. The PMDI preparations kept in a freezer were stable during the whole investigative period (1 year), while all the other preparations failed to fulfil the requirements of stability, i.e. $\pm 20\%$ of the start concentration, which was 1.97% (CI 1.87–2.07) for 4,4'-MDI and 0.58% (CI 0.56–0.60) for PMDI (with respect to the 4,4'-MDI content). The preparations of 4,4'-MDI stored in a freezer were not stable during the whole study period. However, storage in a freezer prolonged the lifetime also for the preparations of pure 4,4'-MDI. Moreover, the decrease in concentration for the preparations stored at room temperature and in a refrigerator seems to be less rapid in the preparations containing PMDI than in the ones containing pure 4,4'-MDI. As

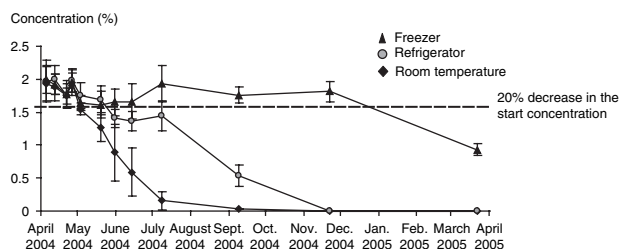


Fig. 3. Stability of pure diphenylmethane-4,4'-diisocyanate in petrolatum under varying storage conditions for 1 year. Each dot represents the mean of 9 samples (i.e. triple samples from 3 different preparations stored under the same condition) $\pm$ standard deviation. The dotted line represents a 20% decrease in the initial amount of sensitizer present at time 0.

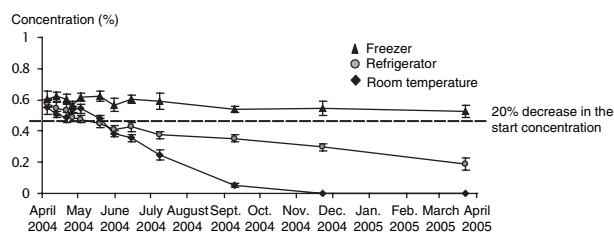


Fig. 4. Stability of the diphenylmethane-4,4'-diisocyanate content in petrolatum preparations of the polymeric diphenylmethane diisocyanate product Pedilen under varying storage conditions for 1 year. Each dot represents the mean of 9 samples (i.e. triple samples from 3 different preparations stored under the same condition) $\pm$ standard deviation. The dotted line represents a 20% decrease in the initial amount of sensitizer present at time 0.

shown in Fig. 5, the decrease in the concentration of the 3- and 4-ring oligomers in PMDI declines in approximately the same way as the concentration of 4,4'-MDI.

Statistical analyses using the Mann–Whitney test showed that the variation of triple samples of 4,4'-MDI was significantly higher than the variation of triple samples of PMDI ($p = 0.02$).

The C.V. for the 10 samples analysed to determine the repeatability of the method with respect to 4,4'-MDI in pet. patch-test preparations of Pedilen was 3.3%.

Discussion

The elicitation of a positive patch-test reaction depends among other things on the dose, which is determined by the concentration and volume/amount of test preparation applied as well as on the patch test technique, i.e. the vehicle used and type of occlusion (12–14). To test patients with a dose that elicits a positive patch-test reaction, it is of outmost importance that the patch-test preparation is stable and that the allergen is evenly distributed in the vehicle so that the concentration is known and kept the same throughout the whole preparation. Pet. is the preferred patch-test vehicle for most contact sensitizers, and although exceptions have been reported (8, 15–18), it has been shown that many compounds prepared in pet. are stable for years when kept in a refrigerator at 4°C (19). Isocyanates are highly reactive chemicals and their stability in pet. patch-test preparations has been discussed previously. Estlander et al. (20) concluded that 4,4'-MDI-preparations could be used for over a year after having tested 1 patient with a patch-test preparation of 1.5% 4,4'-MDI in pet. manufactured 5.5 months prior to the testing, and 1 patient with a preparation manufactured 15.5 months earlier, and where both preparations elicited positive patch-test reactions.

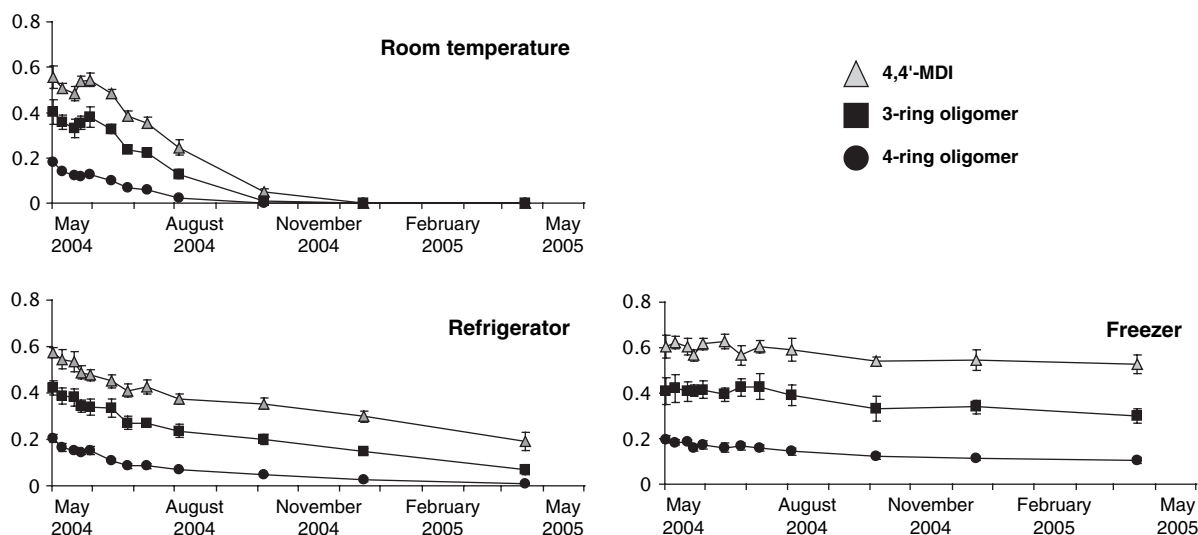


Fig. 5. Comparison of the stability of diphenylmethane-4,4'-diisocyanate, 3-ring oligomers and 4-ring oligomers in the polymeric diphenylmethane diisocyanate product Pedilen under varying storage conditions for 1 year. Each dot represents the mean of 9 samples (i.e. triple samples from 3 different preparations stored under the same condition) $\pm$ standard deviation.

However, no chemical analyses were performed. In 2004, we performed chemical analyses of 14 commercial test preparations of 4,4'-MDI obtained from 8 European and 4 American dermatology departments and from 2 major European suppliers of patch-test allergens. It was shown that none of these could be considered stable (8). The criterion for stability was $\pm 20\%$ of the start concentration.

The aforementioned findings caused us to investigate the stability of pet. patch-test preparations of 4,4'-MDI over time when stored under 3 different conditions, namely at room temperature, in a refrigerator and in a freezer. Commercially available patch-test preparations of MDI, such as those investigated in the above-mentioned study (8), contain only 1 isomer i.e. 4,4'-MDI. This substance consists of solid flakes, which need to be ground before it can be dispersed into pet. When commercially available preparations of 4,4'-MDI was previously analysed, it was concluded that there was a slight variation between triple samples taken from the same preparation. This variation could not be explained by methodological deficiencies, with regard to the analytical procedure. Instead, it was suggested that the preparations were inhomogeneous, probably because of difficulties in dispersing 4,4'-MDI into pet. or because of differences in the degree of possible polymerization in different parts of the syringe (8). Since PMDI is a viscous liquid which is easily dissolved in pet., we decided to investigate the stability of pet. patch-test preparations of PMDI as well. As suspected, the present study shows that the variation between triple samples (with regard to the 4,4'-MDI content) is significantly less

($p = 0.02$) in preparations containing PMDI than in those containing pure 4,4'-MDI (Figs 3 and 4). This strengthens the proposition that difficulties in dispersing 4,4'-MDI in pet. lead to inhomogeneous preparations (8).

Furthermore, it is shown that it is preferable to use pet. preparations of PMDI when patch testing for contact allergy to isocyanates since they are more stable than preparations containing 4,4'-MDI. In fact, it was only the preparations of PMDI stored in a freezer that kept stable (i.e. concentration $\geq 80\%$ of the initial concentration) during the whole study period. The 4,4'-MDI preparation stored in freezer had, on the other contrary, decreased to 80% of the initial concentration at approximately 8.5 months. At the end of the investigative period (1 year), the concentration had declined over 50% of the initial concentration. The same pattern, i.e. that preparations of PMDI are more stable than those of 4,4'-MDI, was also seen for the syringes kept at room temperature and in a refrigerator. Although none was stable for more than about 1.5 to 2 months, the decline in concentration over the entire investigative period was less rapid in the preparations containing PMDI (Figs 3 and 4). The content of 3- and 4-ring oligomers in Pedilen was also monitored. They seem to decrease in the same rate as 4,4'-MDI (Fig. 5). When investigating the behaviour of polymeric MDI in water, Yakabe et al. reported that solid and insoluble polyurea is formed, which encrusts the isocyanate and gradually spreads through it (21). The decrease in 4,4'-MDI as well as in 3-ring oligomers and 4-ring oligomers probably depends on this kind of formation of polyureas after reaction with water

in the air and in the syringes. These polyureas have a high molecular weight and should thus not be considered potential contact allergens. It is not yet known why pure 4,4'-MDI polymerizes in a higher rate than 4,4'-MDI present in PMDI does.

The use of PMDI instead of 4,4'-MDI when patch testing for contact allergy to isocyanates is further fortified by the fact that PMDI better reflects the possible allergens that workers are in fact exposed to. Polyisocyanate-based products such as PMDI, which contains several monomeric isomers and oligomers (Table 1) are the major contributors of exposure to isocyanates in many work environments. However, commercially available preparations only contain 1 monomeric isomer, in this case, 4,4'-MDI. It is not yet known if 4,4'-MDI is the most potent allergen in PMDI or if there are other monomers and/or oligomers that can be the cause of skin problems among exposed workers. In a not yet published study, we patch tested 12 diaminodiphenylmethane allergic individuals, of which 4 had a known contact allergy to 4,4'-MDI and/or work material containing PMDI, with 3 different kinds of MDI products (2 products with the composition characteristic for PMDI and 1 product consisting of the 2 monomeric isomers 4,4'-MDI and 2,4'-MDI) and with pure 4,4'-MDI that was freshly made. 9 of 12 reacted to 1 or more of the MDI-products. 3 of these 9 did not react to pure 4,4'-MDI, and with 2 exceptions the reactions to 4,4'-MDI in the remaining 6 cases were much weaker than the reactions to at least 1 of the MDI-products (22). Consequently, in most cases, patch testing with PMDIs and/or a product with a high 2,4'-MDI content elicited stronger reactions than what patch testing with pure 4,4'-MDI did. Since the concentration of 4,4'-MDI in the MDI-products was less than half of the concentration in pure 4,4'-MDI, these preliminary results further indicate that more allergens beside 4,4'-MDI might be present in PMDI products.

Conclusions

Pet. preparations of PMDI with a composition resembling the one investigated here are better suited to use when patch testing patients exposed to MDI because they are more stable and more homogeneous than those of 4,4'-MDI. They also better reflect the possible allergens to which workers are in fact exposed to because the products used in industry often consist of complex mixtures containing both monomeric diisocyanates and polyisocyanates of different sizes. However, because the composition of PMDIs varies with manufacturer, it is not possible to give a general

recommendation that PMDIs always are better to use than 4,4'-MDI without doing a chemical characterization of the PMDI in question first.

Acknowledgements

The authors thank Marianne Dalene and Viveca Bursius at Work Environment Chemistry, Stockholm University, Håssleholm, for valuable assistance and the Swedish council for working life and social research for providing funds.

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