

Factors Affecting the Concentration of Diphenylmethane-4,4'-Diisocyanate in Freund's Complete Adjuvant. Can They Affect the Outcome of the Guinea-Pig Maximization Test?

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Abstract

Background: The animal assay Guinea Pig Maximization Test (GPMT) uses Freund's complete adjuvant (FCA) to enhance the dermal sensitization in the animals. In a previous study, the degree of sensitization when investigating diphenylmethane-4,4'-diisocyanate (4,4'-MDI) varied. 4,4'-MDI is known to be a reactive diisocyanate and concern was risen that its instability and reaction with FCA might affect the outcome.

Objectives: To investigate the stability of 4,4'-MDI and its possible reaction with components in FCA.

Methods: The same preparations as used in the GPMT were prepared, i.e. 1.0% w/v 4,4'-MDI in FCA/paraffin oil (40/60 v/v) and stored under different conditions for 48 hours. The content of 4,4'-MDI in both FCA/paraffin oil mixtures as well as in samples of pure substance were monitored by chemical analysis using gel permeation chromatography (GPC).

Results: The 4,4'-MDI content in pure substance can decrease substantially within a few months. A rapid decrease in 4,4'-MDI concentration was also seen in 4,4'-MDI/FCA preparations stored in refrigerator and room temperature.

Conclusion: The outcome of 4,4'-MDI sensitization in GPMT might be affected by the instability of the pure substance as well as its reaction with FCA. Major effects can be expected if these two factors are interacting. Hence, fresh 4,4'-MDI should be used and preparations of induction substances should be prepared in close connection to the intradermal injection.

Keywords: Freund's complete adjuvant; FCA, diphenylmethane-4,4'-diisocyanate; 4,4'-MDI; Guinea Pig Maximization Test; GPMT

Introduction

The Guinea Pig Maximization Test (GPMT) is a well-established method used to detect allergens and their cross-reaction patterns. The GPMT relies on using Freund's Complete Adjuvant (FCA) to enhance the sensitization. Recently, we conducted a study in which the sensitizing capacity and cross-reactivity of the aromatic diisocyanate diphenylmethane-4,4'-diisocyanate (4,4'-MDI) was investigated using the GPMT [1]. In that study, 4,4'-MDI was used as the induction substance on three different occasions. The induction phase includes intradermal and epidermal sensitization, which is followed by the elicitation phase. In the induction phase, the animals are shaved at the neck and receive three intradermal injections on each side of the shoulder. FCA is used in two of these injections, one of which FCA is mixed with 4,4'-MDI and the vehicle of choice. The induction phase has been previously described in [1]. Two weeks after completed induction, the animals are patch tested with the induction substance. Two days later the animals are read to identify sensitized animals. On the first occasion, 4,4'-MDI was shown to be a strong sensitizer inducing reaction in 18 out of 24 animals tested. However, on the second

occasion the induction failed and only 2 out of 24 test animals were tested positive to 4,4'-MDI. 4,4'-MDI was used as an induction substance a third time, where 8 out of 24 test animals reacted, rendering 4,4'-MDI a weak allergen based upon the set criterions [2].

After reviewing the different steps in the sensitization process, two main possible factors could explain the varying degrees in sensitization found: 1) the age of the 4,4'-MDI used, and 2) 4,4'-MDI might react with components in FCA.

The aim of the study was to investigate if the content of 4,4'-MDI in preparations used for induction of sensitization in the GPMT is decreased by mixing it with FCA and if the decrease is influenced by the storage temperature, the time lap between preparation and injection as well as the age of the 4,4'-MDI.

Materials and Methods

Reagents

The chemicals used were the following; 4,4'-MDI (Sigma-Aldrich GmbH, Steinheim, Germany), Imject™ Freund's complete adjuvant (Thermo Scientific, Rockford, USA), paraffin oil (APL, Stockholm,

Sweden), and dichloromethane HyperSolve Chromanorm (VWR Chemicals, Fonteney-sous-Bois, France).

Sample preparation

Two preparations, approximately 6 ml each of 1.0% w/v 4,4'-MDI in a vehicle consisting of FCA and paraffin oil in the ratio 40/60 v/v were prepared. One was placed in the refrigerator (8°C) and one was stored in room temperature (25°C). 4,4'-MDI was first dissolved in FCA and then further diluted in paraffin oil for the intended concentrations, i.e. 1.0% w/v 4,4'-MDI in FCA/paraffin oil (40/60 v/v). Samples were taken from each emulsion at 0, 2, 4, 6, 24 and 48 h. Double samples of about 100 mg were dissolved in 25.0 ml dichloromethane and analyzed in triplicates.

4,4'-MDI batches

Three different batches of 4,4'-MDI were analyzed to state the concentration of 4,4'-MDI found in each package. One package of 4,4'-MDI was new and used as reference substance. The second was 4.5 months old and the third one was 3 years and 8 months old. All batches of 4,4'-MDI had been stored in a -21°C freezer. Samples of 4,4'-MDI flakes were mixed with dichloromethane for a solution with an intended concentration of 1.0% w/v and ultra-sonicated for 5 minutes. The solutions were filtered when needed. The samples were diluted 100 times and analyzed by gel permeation chromatography.

Gel Permeation Chromatography (GPC)

The analysis was carried out using a system consisting of an L-2200 auto sampler, an L-2130 pump and an L-2455 diode array detector (La Chrome Elite, Hitachi High-Technologies Corporation, Tokyo, Japan). The system was controlled by EZCHROM ELITE software (Agilent Technologies, Inc., CA, USA). A GPC guard column TSK guard column HHR-L (6 mm internal diameter × 4 cm and particle size 13 μm) connected to a GPC column TSK-Gel® G1000HHR Column (7.8 mm internal diameter × 30 cm, polystyrene, particle size 5 μm and exclusion limit 1 × 10³) was used. The stationary phase consisted of polystyrene crosslinked with divinylbenzene and the mobile phase consisted of dichloromethane. The injection volume was 20 μl and the flow rate was 1.0 ml/min. The detector was set at a wave length of 238 nm for the detection of 4,4'-MDI.

Results

The mean value of triplicate samples of each double sample collected from the preparations was calculated and plotted. A concentration decreases of 4,4'-MDI during the 48 hours could be seen for both storage conditions (Figures 1 and 2). As expected the decrease was more rapid in room temperature where the concentration of 4,4'-MDI had declined to 0.33% w/v after 48 hours while the corresponding concentration in refrigerator after 48 hours was 0.67% w/v.

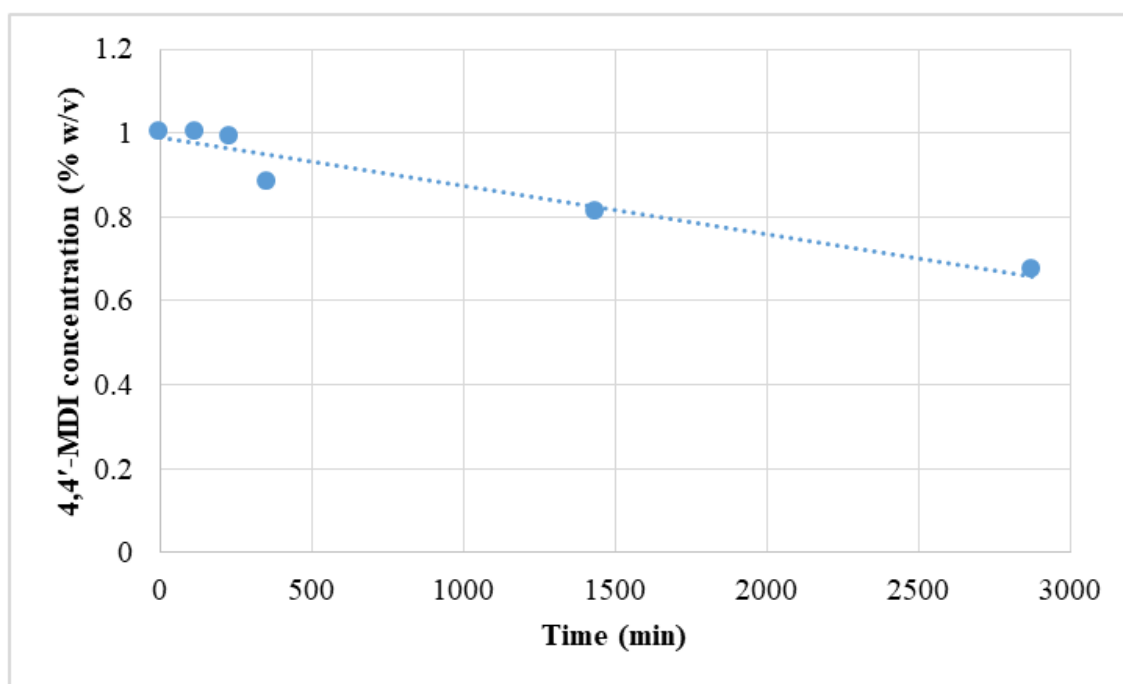


Figure 1: Diphenylmethane-4,4'-diisocyanate (4,4'-MDI) in Freund's complete adjuvant/paraffin oil stored in refrigerator at 8°C. Method of preparation: diphenylmethane-4,4'-diisocyanate dissolved in Freund's complete adjuvant and emulsified in paraffin oil.

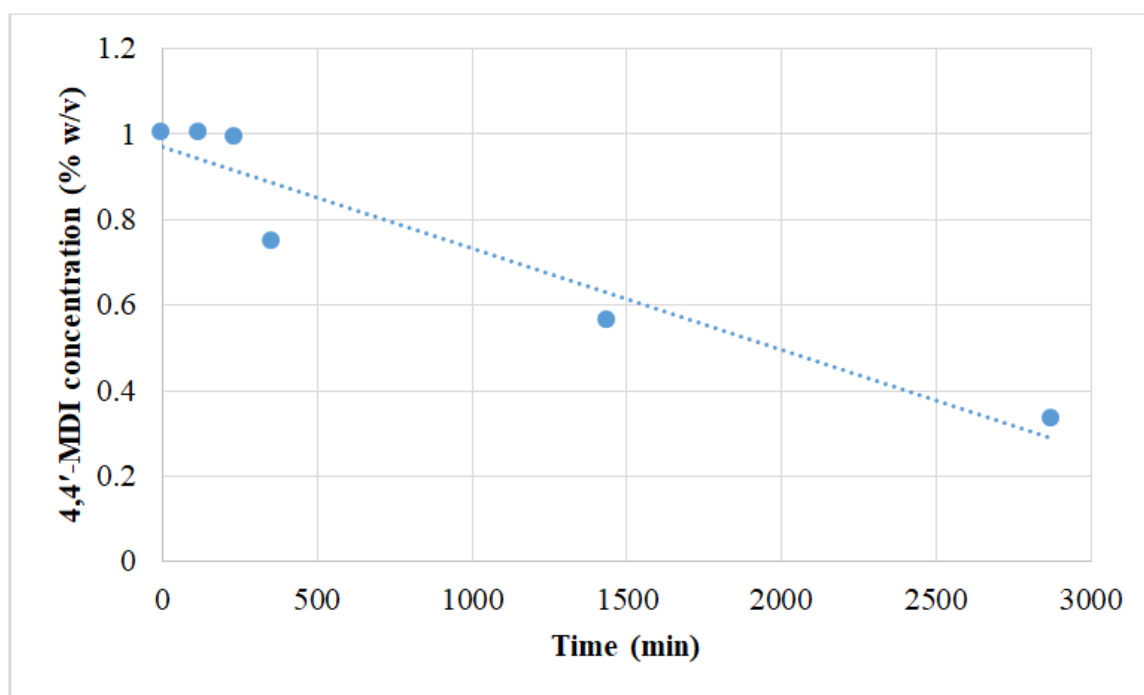


Figure 2: Diphenylmethane-4,4'-diisocyanate (4,4'-MDI) in Freund's complete adjuvant/paraffin oil stored in room temperature at 25°C. Method of preparation: diphenylmethane-4,4'-diisocyanate dissolved in Freund's complete adjuvant and emulsified in paraffin oil.

In comparison with a fresh and newly opened batch of 4,4'-MDI, which was used as reference substance it was shown that the 4.5 months old batch contained 57% w/w 4,4'-MDI and the 3,7 years old batch contained 42% w/w 4,4'-MDI (Table 1).

4,4'-MDI age (months)	Concentration (% w/w)
0 (reference substance)	100
4.5	57
44	42

Table 1: Diphenylmethane-4,4'-diisocyanate (4,4'-MDI) concentrations in different batches.

Discussion

GPMT is widely used to investigate contact allergens and was first developed by Magnusson and Kligman in 1969 and has been described extensively in previous studies [1-4]. It is an established method to detect sensitizers and their cross-reaction patterns [5]. In the 1980's the method was modified by Bruze in order to standardize it making it possible to statistically evaluate the patch test reactions, where blind reading and a positive control group were introduced [6,7]. The method uses FCA to enhance the sensitization in the intradermal part of the induction phase. The induction phase, consists of an intradermal sensitization part performed on day 0 followed by an epidermal sensitization part on D7 and is described in detail in the original article by Magnusson and Kligman [1].

The first part of the induction phase includes three intradermal injections in a row on each side of the shoulder. Of the three injections made on each side of the shoulder, FCA is used in two. In one injection FCA and the vehicle is used and in the second FCA, vehicle and the investigated substance is used [2]. A third injection is made on each side containing the investigated substance in the vehicle of choice. Since isocyanates are highly reactive with alcohols, paraffin oil was used as vehicle instead of the more common choice propylene glycol.

In the previous study, where the GPMT was used to investigate the sensitizing capacity and cross-reactivity of 4,4'-MDI the sensitization gave different results when performed at three different occasions, namely strong sensitizer, no sensitization and weak sensitizer [2]. The sensitization procedure was investigated for errors but no obvious reasons for the different results were found [2]. The positive controls reacted as expected. However, it was suggested that one plausible explanation could be a variation of the 4,4'-MDI content in the preparations used in the intradermal sensitization. Therefore, the preparations containing 4,4'-MDI which had been used in the intradermal induction at the third and last sensitization trial with 4,4'-MDI were analyzed. The result showed that the preparation containing 4,4'-MDI and paraffin oil was stable, while the other containing FCA, 4,4'-MDI and paraffin oil reduced 4,4'-MDI concentration. Therefore, we investigated the stability of 4,4'-MDI when mixed with FCA. Mixtures of this type were analyzed over time after storage in refrigerator and at room temperature. Another factor that might affect the outcome is the stability of the raw material, i.e. 4,4'-MDI. Therefore, 4,4'-MDI of different ages were also investigated.

4,4'-MDI is a reactive substance and reacts readily with water, amino-, thiol- and hydroxyl groups in other substances. In FCA there

are proteins and water which are targets for 4,4'-MDI due to its reactivity with amino- and hydroxyl groups. FCA contains water, oil, emulsifier and killed *Mycobacterium* bacteria. At the start of this study the 4,4'-MDI preparation was prepared according to the guidelines that had been used in the previous GMPT studies. These guidelines stated dissolving 4,4'-MDI in FCA and later mixing with the vehicle of choice [2,8].

In all the three sensitization trials of 4,4'-MDI the induction emulsion with FCA was made one day prior to the intradermal sensitization and stored in a refrigerator. The emulsion was then taken out of the refrigerator after approximately 24 h, delivered to the animal testing laboratory, and stored at room temperature for approximately 6 hours while the animals were being prepared for the induction phase. The results show that storage conditions play a vital role. When stored at room temperature the concentration of 4,4'-MDI in the mixture was 0.56% w/v after 24 h from the start of the preparation. At the end of the assay (48h), only 0.33% w/v remained in the emulsion. For the mixture stored in refrigerator the concentration was 0.81% w/v after 24 h and after 48 hours the concentration of 4,4'-MDI was 0.67% w/v (Figures 1 and 2). The decrease of 4,4'-MDI concentration could affect the sensitization process. As a conclusion from the above results, the approximate concentration of 4,4'-MDI in the intradermal FCA-preparations that were used could be extrapolated from Figures 1 and 2 to around 0.61% w/v compared to the intended concentration i.e. 1.0% w/v.

Analysis of 4,4'-MDI of different ages shows that pure 4,4'-MDI is a very reactive chemical. Despite being stored in -21°C the concentration decreases substantially in just few months (Table 1). This might have a major impact on the outcome of the method since it might affect both the intradermal and epidermal induction as well as the elicitation.

The use of FCA in GPMT is a generally sensitive method for characterizing and identifying contact allergens by enhancing the sensitization in guinea pig but it is important to point out that reactive chemicals might not be at the intended concentration in the preparations used. Based on our findings the induction emulsion in FCA should be prepared in direct connection to the administration of the intradermal injection of the substance. Furthermore, it is paramount to use new 4,4'-MDI to guarantee correct concentrations in both induction and elicitation. In similar assays for other aromatic diisocyanates or other highly reactive substances, it may be of interest to compare the intended and the actual injected concentration.

Conclusion

The outcome of 4,4'-MDI sensitization in GPMT might be affected by the instability of the pure substance as well as its reaction with FCA

constituents. Major effects can be expected if these two factors are interacting. Hence, fresh 4,4'-MDI should be used and induction substances should be prepared in close connection to the intradermal injection.

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Conflicts of Interest

The authors declare no conflict of interest.

Author contributions

All authors have participated sufficiently to take public responsibility for the work.

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