

Sensitizing Capacity of 4,4¹-Dihydroxy-(Hydroxymethyl)-Diphenyl Methanes in the Guinea Pig

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The sensitizing capacity of 4,4¹-dihydroxy-3-(hydroxymethyl)-diphenyl methane (4,4¹-H-3-HPM), 4,4¹-dihydroxy-3,3¹-di-(hydroxymethyl)-diphenyl methane (4,4¹-H-3,3¹--HPM) and 4,4¹-dihydroxy-3,5-di-(hydroxymethyl)-diphenyl methane (4,4¹-H-3,5-HPM) was investigated using the guinea pig maximization test. These compounds are known contact sensitizers in phenol-formaldehyde resins (P-F-R). The study was performed in order to assess and compare the degrees of the sensitizing capacities of these chemically related substances. The animals were also rechallenged with the sensitizer and 5 related compounds, all known to be present in P-F-R, in order to study the cross-reaction patterns. 4,4¹-H-3,3¹-HPM was demonstrated to be a strong sensitizer and 4,4¹-H-3-HPM and 4,4¹-H-3,5-HPM to be moderate sensitizers. With 4,4¹-H-3,3¹-HPM as the sensitizer, 4,4¹-H-3-HPM and 4,4¹-H-3,5-HPM were cross-reacting substances and simple methylol phenols were possible cross-reacting compounds. Possible cross-reactivity were indicated between the three 4,4¹-H-HPM when 4,4¹-H-3-HPM and 4,4¹-H-3,5-HPM respectively were the sensitizers. The chemical investigation by high pressure liquid chromatography indicated that the compounds tested were pure and separable. *Key words: Delayed hypersensitivity; Guinea pig maximization test; High pressure liquid chromatography; Phenol-formaldehyde resins.* (Received September 3, 1985.)

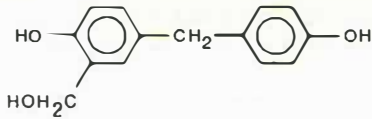
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In recent years several new contact sensitizers in resins, based on phenol and formaldehyde (P-F-R) have been recognized (1-5). The 4,4¹-dihydroxy-(hydroxymethyl)-diphenyl methanes (4,4¹-H-HPM) seem to be the most potent sensitizers of those isolated and identified according to the results of patch testing in subjects already shown to be P-F-R sensitive. It is, however, impossible to assess the degree of sensitizing capacity of a sensitizer in P-F-R, in subjects already sensitized to P-F-R. It is also impossible to base differences in sensitizing capacity of chemically related compounds on comparisons in these subjects. Both these points may, on the other hand, be elucidated by predictive patch testing in animals, which are sensitized to each ingredient separately. The purpose of this study was, therefore, to determine the sensitizing capacities of three 4,4¹-H-HPM and also to compare the sensitizing capacities and investigate the cross-reaction patterns by using guinea pigs for the sensitization.

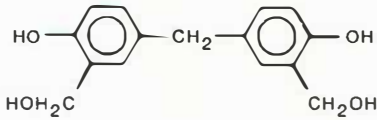
MATERIAL AND METHODS

Substances

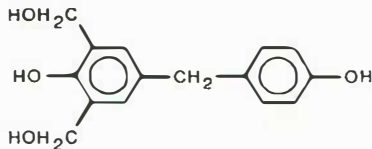
Induction was performed with 4,4¹-dihydroxy-3-(hydroxymethyl)-diphenyl methane (4,4¹-H-3-HPM), 4,4¹-dihydroxy-3,3¹-di-(hydroxymethyl)-diphenyl methane (4,4¹-H-3,3¹-HPM) and 4,4¹-dihydroxy-3,5-di-(hydroxymethyl)-diphenyl methane (4,4¹-H-3,5-HPM). These compounds were synthesized at the department and identified by mass-spectrometry and nuclear magnetic resonance spectrometry (5). Challenges were performed with these 4,4¹-H-HPM and also with 2-methylol phenol (2-MP) (Merck, West Germany), 4-methylol phenol (4-MP) (Merck), 2,4,6-trimethylol phenol (2,4,6-MP) and



4,4'-dihydroxy-3-(hydroxymethyl)-
-diphenyl methane



4,4'-dihydroxy-3,3'-di-(hydroxymethyl)-
-diphenyl methane



4,4'-dihydroxy-3,5-di-(hydroxymethyl)-
-diphenyl methane

Fig. 1. The structural formulae of 4,4'-dihydroxy-3-(hydroxymethyl)-diphenyl methane, 4,4'-dihydroxy-3,3'-di-(hydroxymethyl)-diphenyl methane and 4,4'-dihydroxy-3,5-di-(hydroxymethyl)-diphenyl methane.

2,6-dimethylol phenol (2,6-MP). The latter substances were synthesized at the department and identified by mass-spectrometry and nuclear magnetic resonance spectrometry. The structural formulae of the compounds used for the inductions are shown in Fig. 1.

High Pressure Liquid Chromatography (HPLC)

All the substances used for inductions and challenges were analysed by HPLC on a bonded octadecylsilylphase using methanol (Merck, Lichrosolv)/water as the mobile phase and detected by a UV-detector. All analyses were performed using a column (20 cm, 4 mm i.d.) packed with Nucleosil C₁₈ (5 µm, Macherey-Nagel & Co., West Germany). The samples were dissolved in the mobile phase. The flow rate was 1 ml/min and the eluate monitored at 280 nm by using an LDC-spectroMonitor D, variable wavelength detector.

The "Guinea pig maximization test" (GPMT) was performed in accordance with the original descriptions (6-8) but with some modifications in order to increase the standardization of the test and also to create conditions for objective evaluation, including statistical calculations of the patch test reactions. These modifications implied, briefly, that the same number of molecules of a substance was administered to the animals when the figures for the concentrations were the same independent of the vehicle used. The distribution of test and control animals randomly to the cages, and the judgment of the test results based on statistical comparisons, are other modifications of the GPMT.

Animals

Albino female guinea pigs of the Dunkin-Hartley strain (J A Sahlin, Sweden) weighing 300-400 g were used. For each one of the 4 sensitization series (2 series with 4,4'-H-3-HPM and 1 series for each of the other two 4,4'-H-HPM) 36 animals were used; 12 in the control group and 24 in the test group. The animals included in these procedures were not engaged in tests for topical irritancy.

The topical irritancy of the substances used for inductions and challenges was studied by a 48-hour

closed patch test in 4-8 animals for each compound. On each animal the compound was applied on 3 patches on the flank; one near the back, one near the abdomen and one between these.

Induction procedure

4,4'-H-3-HPM, 4,4'-H-3,3'-HPM and 4,4'-H-3,5-HPM were used for sensitization. For intradermal sensitization 3 injections were given in a row, on each side of the shoulder. (I). 0.1 ml Freund's complete adjuvant (FCA) (Difco Lab. USA) in water 40% w/v (corresponds to FCA/water 50/50 v/v). (II). 0.1 ml of 4,4'-H-3-HPM or 4,4'-H-3,3'-HPM or 4,4'-H-3,5-HPM. The concentrations used were equimolar (3.8×10^{-3} mole $\times 1^{-1}$) and 0.88% w/v for 4,4'-H-3-HPM and 1.00% for 4,4'-H-3,3'-HPM and 4,4'-H-3,5-HPM. The vehicle was propylene glycol. (III). 0.1 ml of the preparation consisting of the potential sensitizer (4,4'-H-3-HPM, 4,4'-H-3,3'-HPM, 4,4'-H-3,5-HPM)/FCA/propylene glycol w/w/v. The figures for the concentrations were the same as for (I) and (II).

24 h before the topical sensitization all the animals were treated with sodium lauryl sulphate (SLS) 10% w/v in dimethylacetamide/acetone/ ethanol 99.5% 4/3/3 v/v/v (DAE 433). Equivalent results, concerning the sensitizing capacity of diglycidylether of bisphenol A, were obtained with this vehicle for SLS when compared to SLS in petrolatum (to be published). DAE 433 facilitates the application of a desired volume (200 μ l) to a limited area and the inflammation obtained is moderate and of the same degree all over the application area. 200 μ l of the suspected sensitizer in acetone/ethanol 99.5% 1/1 v/v, at a concentration of 2.21% w/v for 4,4'-H-3-HPM and 2.50% w/v for 4,4'-H-3,3'-HPM and 4,4'-H-3,5-HPM, was transferred to a 2 \times 4 cm patch of Whatman 3MM filter paper. The concentrations were equimolar (9.6×10^{-3} mole $\times 1^{-1}$). The patch was covered with overlapping, impermeable plastic adhesive tape (Leukoflex, Beiersdorf AG, West Germany). This in turn was firmly secured by an adhesive bandage (Acrylastic, Beiersdorf AG). The dressing was left in place for 48 h.

Challenge procedure

Two weeks after the second stage of sensitization a 24-hour occluded patch test (AI-test, on Leukoflex and firmly secured by Acrylastic) was performed on the right flank with 30 μ l of the test solution on each of 2 patches near the back. In each sensitization study 12 animals received the suspected sensitizer in acetone/ethanol 99.5% 1/1 v/v on both patches. Half the number of animals (6) received the suspected sensitizer on only one of the patches while the vehicle alone was applied to the other patch. The same number of animals received the suspected sensitizer and the vehicle in the reverse way. All control animals received the suspected sensitizer on both patches. The concentrations used were equimolar (7.7×10^{-3} mole $\times 1^{-1}$); 1.77% w/v for 4,4'-H-3-HPM and 2.00% for 4,4'-H-3,3'-HPM and 4,4'-H-3,5-HPM.

Rechallenge was performed one week after the challenge according to the technique previously described (9). The animals in the first series with 4,4'-H-3-HPM were not rechallenged. 0.1 ml of the solutions described in (II) in the section of "induction procedure" was injected intradermally in the neck two days after the first challenge application. Five days later (one week after the first challenge application) the animals were rechallenged with the sensitizer and 5 chemically related substances, which were applied to the left, non-tested flank. The same positions as for the challenge were used and also two positions near the abdomen and the remaining two positions between the back and the abdomen. A distribution pattern, based on a Latin square table, was used for the rechallenge. The sensitizer and each one of the 5 chemically related substances were applied twice in each position on the control animals and the corresponding figure for the test animals was 4. The animals were rechallenged with all substances in acetone/ethanol 99.5% 1/1 v/v at equimolar concentrations (7.7×10^{-3} mole $\times 1^{-1}$). With 4,4'-H-3-HPM as the sensitizer rechallenge was carried out with all three 4,4'-H-HPM and also with 2-MP 0.95% w/v, 4-MP 0.95% w/v and 2,4,6-MP 1.42% w/v. The same substances were tested with 4,4'-H-3,3'-HPM and 4,4'-H-3,5-HPM as the sensitizers, except the exchange of 2,4,6-MP with 2,6-MP 1.18% w/v when 4,4'-H-3,5-HPM was the sensitizer.

Controls

The animals in each control group were treated in the same way, concerning the induction and challenge procedures, as corresponding animals in the test group except that the suspected sensitizer was not administered during the induction and in the booster dose before rechallenge.

Evaluation

The reactions were evaluated blind. The minimum criterion of an allergic (positive) reaction was a confluent erythema (6-8).

The number of positive animals in each test group was statistically compared to the number of positive animals in the corresponding control group and also to the number of positive animals tested with the vehicle alone. The assessment of whether an animal was positive or not, was based on the

Table I. Test reactions after sensitization to and challenge with 4,4'-dihydroxy-3-(hydroxymethyl)-diphenyl methane (4,4'-H-3-HPM), 4,4'-dihydroxy-3,3'-di-(hydroxymethyl)-diphenyl methane (4,4'-H-3,3'-HPM) and 4,4'-dihydroxy-3,5-di-(hydroxymethyl)-diphenyl methane (4,4'-H-3,5-HPM)

C=control animals, T=test animals receiving the suspected sensitizer, V=test animals receiving the vehicle alone, n=number of tested animals in the 3 groups—C, T, V—in each series

Animal group	Number of positive animals		
	C n=12	T n=24	V n=12
4,4'-H-3-HPM			
Series 1	0	5	0
Series 2	0	17	0
4,4'-H-3,3'-HPM			
Series 1 ^a	0	20	1
4,4'-H-3,5-HPM			
Series 1	0	11	0

^a One test animal died during induction so only 11 animals obtained the suspected sensitizer on both patches when challenged.

result for only one patch chosen in advance for those animals which had obtained the test solution on both patches (all 12 control animals and 12 test animals). When both comparisons yielded significant values the compound was considered to be a contact sensitizer. The significance levels (the lowest level was chosen when not identical) $p < 0.05$; $p < 0.01$; $p < 0.001$ were used to designate a weak, moderate and strong sensitizer respectively.

For the rechallenge a comparison was made only between the number of positive animals in the test and control groups for each substance.

Statistical calculation

Fisher's exact test for two proportions was used.

RESULTS

The highest possible contaminations of the 4,4'-H-HPM in the compounds used for challenges and rechallenges, were below 0.60% w/w for all substances.

Table I shows the results of the sensitization to and challenge with 4,4'-H-3-HPM, 4,4'-H-3,3'-HPM and 4,4'-H-3,5-HPM. The difference in the number of positive animals for 4,4'-H-3-HPM between the test and control groups was non-significant. However, when the sensitization was repeated with a new series, 17 test animals were positive and no controls reacted. Nor did the test animals react to the vehicle alone and both comparisons were statistically significant ($p < 0.001$). The significance level was the same when the results for the series were added but lower ($p < 0.01$) when the calculations were adjusted to the number of animals in one series.

Patch testing with 4,4'-H-3,3'-HPM and 4,4'-H-3,5-HPM also implied more animals reacting in the test groups compared to the control groups. No controls reacted at all and only one animal in the 4,4'-H-3,3'-HPM test group, reacted to the vehicle alone. The differences between the number of positive animals in the test and control groups were significant for both 4,4'-H-3,3'-HPM ($p < 0.001$) and 4,4'-H-3,5-HPM ($p < 0.01$). Significant differences were also noted within the test groups, for both compounds, when the

Table II. Test reactions after rechallenge with 4,4'-dihydroxy-3-(hydroxymethyl)-diphenyl methane (4,4'-H-3-HPM), 4,4'-dihydroxy-3,3'-di-(hydroxymethyl)-diphenyl methane (4,4'-H-3,3'-HPM), 4,4'-dihydroxy-3,5-di-(hydroxymethyl)-diphenyl methane (4,4'-H-3,5-HPM), 2-methylol phenol (2-MP), 4-methylol phenol (4-MP), 2,4,6-trimethylol phenol (2,4,6-MP) and 2,6-dimethylol phenol (2,6-MP)

T=test group, C=control group

Sensitization substance	No. of animals	No. of positive animals after rechallenge with						
		4,4'-H-3-HPM	4,4'-H-3,3'-HPM	4,4'-H-3,5-HPM	2-MP	4-MP	2,4,6-MP	2,6-MP
4,4'-H-3-HPM								
T	24	16	5	3	1	1	1	
C	12	6	2	1	0	0	0	
4,4'-H-3,3'-HPM								
T	23 ^a	17	14	13	4	3	6	
C	12	2	1	2	0	0	0	
4,4'-H-3,5-HPM								
T	24	8	3	10	0	0		1
C	12	1	0	2	0	0		0

^a One animal died during induction.

suspected sensitizer and the vehicle were tested simultaneously on the same animals ($p < 0.001$ for 4,4'-H-3,3'-HPM and $p < 0.01$ for 4,4'-H-3,5-HPM).

Table II shows the results of rechallenge with the sensitizer and compounds with similar chemical structures. The number of positive animals in the test group was lower for 4,4'-H-3,3'-HPM while it was almost the same for 4,4'-H-3-HPM and 4,4'-H-3,5-HPM. The decrease was not due to fewer positive test reactions in the additional test positions.

No significant differences were noted between the number of positive test and control animals for those animals that were tested for cross-reactivity to 4,4'-H-3-HPM. Possible cross-reactivity was, however, indicated to 4,4'-H-3,3'-HPM and 4,4'-H-3,5-HPM. With 4,4'-H-3,3'-HPM as the sensitizer there were cross-reactions to 4,4'-H-3-HPM and 4,4'-H-3,5-HPM ($p < 0.01$ and $p < 0.05$) and possible such reactions to simple methylol phenols (non-significant differences). There were no significant differences for those animals that were tested for cross-reactivity to 4,4'-H-3,5-HPM. Possible cross-reactivity was, however, indicated particularly to 4,4'-H-3-HPM but also to 4,4'-H-3,3'-HPM.

DISCUSSION

The design of the GPMT presented does not exclude "false" positive reactions due to irritancy of the suspected sensitizer or to irritancy and/or sensitization (if used before the challenge) to the vehicle. However, wrong conclusions due to "false" positive reactions are excluded since the judgement whether a compound is a sensitizer or not is based on statistical calculations of the figures obtained for the test animals compared to the figures for appropriate control animals.

The differences noted for 4,4'-H-3-HPM, 4,4'-H-3,3'-HPM and 4,4'-H-3,5-HPM were significant. Furthermore, the sensitizing capacities of these particular 4,4'-H-HPM were supported by the results of the HPLC investigations which indicated that the compounds were pure. According to the classification system suggested, 4,4'-H-3,3'-HPM may be considered to be a strong sensitizer and 4,4'-H-3-HPM and 4,4'-H-3,5-HPM to be moderate sensitizers.

The use of a classification system for designating the sensitizing capacity of a compound investigated by the GPMT is always partly based on qualitative arbitrary limits, which necessitates a statistical work up of the results. A classification system may be practically useful under certain conditions. It is valuable when discussing and comparing various sensitizers. To be entirely meaningful such a comparison should start from the same conditions. It is obvious that the concentrations for the induction and challenge for a "strong" sensitizer may be changed in such a way that the result, instead of indicating a strong sensitizer, will indicate a weak sensitizer or maybe even a nonsensitizer. In this study the GPMT for all three substances investigated, was carried out in the same way concerning the number of molecules administered (equimolar concentrations), the vehicles and the use of SLS for all compounds. For these reasons it was possible to consider 4,4'-H-3,3'-HPM to be the strongest sensitizer of those tested. The differences in sensitizing capacity of the three 4,4'-H-HPM were small and this finding is in good agreement with clinical experience (5).

The number of positive test animals differed significantly between the 2 series with 4,4'-H-3-HPM. The reason for this is not clear but may be due to biological differences in the animals.

In the present study, the testing for cross-reactivity was based on equal conditions concerning the concentrations (equimolar), the applied volumes and the test site localization. 4,4'-H-3-HPM and 4,4'-H-3,5-HPM were demonstrated as cross-reacting substances to 4,4'-H-3,3'-HPM. The simple methylol phenols (2-MP, 4-MP, 2,4,6-MP) were also possible crossreacting compounds to 4,4'-H-3,3'-HPM but not to 4,4'-H-3-HPM and 4,4'-H-3,5-HPM. Possible cross-reactions to these latter two sensitizers were shown to the two 4,4'-H-HPM not used for respective induction. The control animals that reacted to 4,4'-H-HPM after rechallenge might have been sensitized during challenge since no control animals reacted at all after challenge.

In a previous study 2-MP was demonstrated to be a strong sensitizer in the guinea pig (10). It is, however, at present impossible to compare the sensitizing capacity of 2-MP and the 4,4'-H-HPM, since the sensitization procedures and challenges were not performed under equal conditions.

This study established 4,4'-H-3-HPM, 4,4'-H-3,3'-HPM and 4,4'-H-3,5-HPM, all present in P-F-R, as contact sensitizers in guinea pigs as well as demonstrating and indicating cross-reacting substances.

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