

Different physical forms of maleopimaric acid give different allergic responses

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Endo-maleopimaric acid (MPA) is a contact allergen formed when colophonium is “modified” with maleic anhydride or fumaric acid. Previous patch testing showed a higher allergic response to petrolatum (pet.) preparations of MPA in amorphous form compared to MPA in crystalline form. In the present study, the impact of the physical form of MPA on the allergic response was investigated. Since the amorphous form is difficult to standardize, crystalline MPA mechanically incorporated or dissolved in pet. was used. A lower eliciting capacity was obtained from crystalline MPA, compared to that obtained from dissolved MPA, in guinea pigs intradermally induced with MPA. Using ^3H -MPA a $3\times$ difference in the dissolution into synthetic sweat from MPA dissolved in pet., compared to MPA mechanically incorporated, was demonstrated. A difference in bio-availability between dissolved and crystalline MPA could therefore be assumed. Crystalline MPA had a low sensitizing capacity compared to that seen for amorphous MPA in previous studies. The amorphous form of MPA is likely to have a larger surface area than crystalline MPA, with less ordered molecules, resulting in a higher dissolution rate and a greater bio-availability. Modified colophonium exists as amorphous solids and as viscous liquids. Thus, exposure will probably be to non-crystalline MPA and cases of contact allergy could be overlooked when screening with crystalline MPA.

Key words: colophonium; dissolution; elicitation experiment; guinea pigs; maleopimaric acid; rosin; patch test preparation; sensitization experiment; bio-availability; vehicle. © Munksgaard, 2002.

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Most colophonium (rosin) used today is modified by reactions with different chemicals. One of the most common modifications is the Diels-Alder reaction of levopimaric acid, formed from abietic acid in colophonium, with maleic anhydride or fumaric acid (Fig. 1). This type of modified product finds its major application in paper production, where it is used for paper sizing, a process to enhance the hydrophobicity of paper. It is also often found in printing ink in combination with other modifications of colophonium (1, 2).

In our studies on the allergenic activity of modified colophonium, we found that a potent contact allergen, endo-maleopimaric acid (MPA) (Fig. 1), was formed in the Diels-Alder reaction of levopimaric acid with maleic anhydride and also with fumaric acid (3, 4). The amount of MPA in modified colophonium varies, but may be considerable. Analyses of different modified products have revealed contents between 8 and 20% (5). Maleic anhydride-polyol esterified colophonium is used as tackifier in shoe linings. Although modified $2\times$, the product still contains components from un-

modified colophonium, together with MPA, that are able to cause allergic contact dermatitis (6).

MPA in amorphous form was shown to be a potent sensitizer in experimental studies in guinea pigs (3). It was subsequently tested in consecutive dermatitis patients. A large number of positive reactions were observed, but with a large batch-to-batch variation in allergenic activity (7). Spectral analyses showed that all batches were the endo-isomer of maleopimaric acid (Fig. 1) and no impurities could be detected. MPA can exist in a highly crystalline form or as an amorphous solid. Similar phenomena are described for other classes of compounds, e.g., solid hydrocarbons that can exist with their discrete molecules arranged in an orderly way as crystals, or as an amorphous solid with the molecules arranged in a disorderly way (8). We discovered that patches with amorphous MPA gave significantly more positive patch test reactions compared to those with MPA in crystalline form (7) (Table 1).

The aim of the present study was to investigate the impact of the physical form of MPA on the

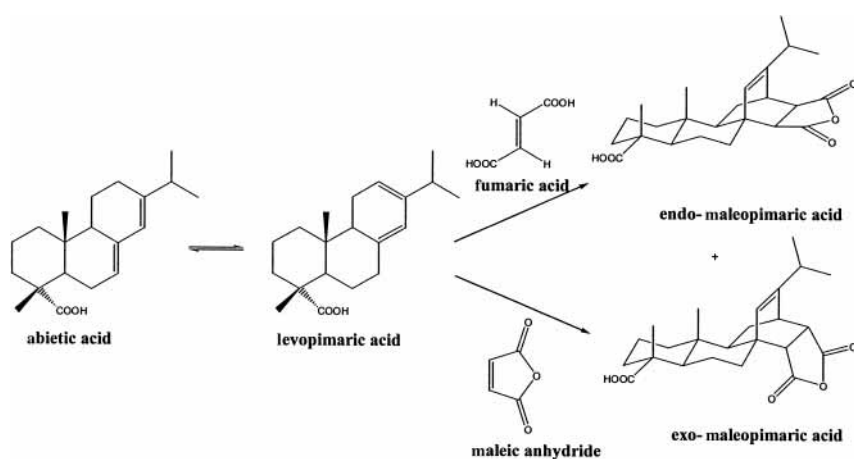


Fig. 1. Maleopimaric acid is formed in a Diels-Alder reaction between levopimaric acid, formed from abietic acid in colophonium and maleic anhydride or fumaric acid. The endo-form is the major product.

allergic response. Attempts were made to relate observed differences in allergenic effect to differences in the dissolution rate of MPA. Since the amorphous form of MPA is difficult to standardize, crystalline MPA mechanically incorporated or dissolved in petrolatum (pet.) was used for additional experiments.

Materials and Methods

Chemicals

MPA was synthesized as earlier described (3). White petrolatum was purchased from Witco Corporation (New York, USA). Freund's complete adjuvant (FCA) was purchased from Difco (Detroit, Mich, USA). Standard chemicals were of analytical or pharmaceutical quality.

Amorphous and crystalline MPA

The amorphous form of MPA was obtained after purification of the crude product with column chromatography eluted with ethyl acetate with increasing amounts of acetic acid (1–10%). The solvent was evaporated leaving a solid material referred to as amorphous MPA. The crystalline ma-

terial was obtained after recrystallization (ether:hexane). Both preparations of MPA had identical spectral properties according to IR, ^1H -NMR and MS.

Synthesis of ^3H -MPA

^3H -MPA was synthesized according to the literature (10). The identification was performed using TLC which showed 1 spot with an identical R_f value to that of authentic MPA. The tritium-labelled maleopimaric acid was crystallized (ether:hexane) and mixed with cold MPA. The specific activity (43,000 dpm/mg) was determined by β -scintillation counting (Beckman LS 6000 TA, USA).

Dissolution of ^3H -MPA from pet. into synthetic sweat

2 different pet. preparations were made, 1 with 1% ^3H -MPA mechanically incorporated into pet. and 1 with 1% ^3H -MPA dissolved in ether and mixed with pet. The ether was then evaporated under a stream of nitrogen. The preparations (1 g) were spread thin on the bottoms of 2 identical beakers (diameter=45 mm); 10 ml synthetic sweat (deionized water containing sodium chloride (0.5%), lactic acid (0.1%) and urea (0.1%), pH 6.5) (10) were added over the pet. layer. The top of the beakers was covered and the beakers were gently shaken for 24 h. Sweat samples (0.5 ml) were collected and transferred to 10 ml of scintillation fluid. The dissolution of MPA was determined by β -scintillation counting.

Experimental sensitization studies

The experiments were approved by the ethics committee. Female outbred Dunkin-Hartley albino

Table 1. Comparison of the patch test result in consecutive dermatitis patients; testing with amorphous or crystalline MPA mechanically incorporated into petrolatum

	No. positive reactions/no. tested ^{a)}		
	amorphous MPA	crystalline MPA	$p^b)$ (amorphous/crystalline)
1.2% in pet.	24/887 (2.7%)	1/185 (0.5%)	<0.01
0.6% in pet.	10/271 (3.7%)	0/146	<0.001

^{a)} The results were obtained in an earlier study (7).

^{b)} Confidence intervals for the difference between proportions were calculated using the normal distribution (9).

guinea pigs weighing 300–350 g, supplied by AB Sahlins Försöksdjursfarm (Malmö, Sweden), were used for the animal experiments. The animals were kept on a standard diet obtained from EWOS AB (Södertälje, Sweden) and water *ad libitum*.

Sensitizing capacity of crystalline MPA. The sensitizing potential of MPA in crystalline form was investigated using the cumulative contact enhancement test (CCET) (12) without FCA injections. The controls were sham-treated and the challenge testing was performed closed, according to our experience (13). A group of 15 animals was induced with MPA in crystalline form, ground finely and mechanically incorporated in pet. Another group of 15 animals formed the control group. The exposed group was treated with 4 closed epidermal applications of crystalline MPA 1.2% (3.0×10^{-5} mol/g) in pet. on the upper back (days 0, 2, 7, 9) for induction. The applications were removed after 24 h. The control group was sham-treated with neat pet. Closed challenge testing was performed on day 21. The test material was applied on the shaved flanks of the animals for 24 h using aluminium test chambers (Finn Chambers®, i.d. 8 mm). The reactions were assessed at 48 h and 72 h after application. The minimum criterion for a positive reaction was a confluent erythema. The animals were challenged with a serial dilution of crystalline MPA mechanically incorporated in pet. The test concentrations were 7.2, 3.6, 1.2, 0.40 and 0.12% (1.8×10^{-4} , 9.0×10^{-5} , 3.0×10^{-5} , 1.0×10^{-5} and 3.0×10^{-6} mol/g). 1 preparation of 7.2% (1.8×10^{-4} mol/g) of amorphous MPA mechanically incorporated in pet. was also tested. The test concentrations were shown in pre-tests to be non-irritant.

Eliciting activity of crystalline and dissolved MPA. The test was performed according to Freund's complete adjuvant test (FCAT) (14) with closed challenge testing according to our experience (13). 30 animals were divided into 1 MPA-exposed group and 1 control group of 15 animals each. On days 0, 6 and 10 the animals were given 1 intradermal injection (0.1 ml) on the upper back for induction. The exposed group was injected with 3.5% (w/w) MPA in FCA/H₂O emulsion (1:1). To enhance the dissolution, the MPA crystals were dissolved in a small amount of acetone before addition of FCA. The acetone was then evaporated with a stream of nitrogen and the emulsion with H₂O was prepared. The control group was sham-treated with an FCA/H₂O emulsion. Closed challenge testing was performed on day 21 as described above. The animals were challenged with 2 serial dilutions of MPA. Series 1 consisted of MPA crystals mechanically incorporated in pet. in test concentrations 1.2, 0.40 and 0.13% (corresponding to

3.0×10^{-5} , 1.0×10^{-5} and 3.2×10^{-6} mol/g). Series 2 consisted of MPA crystals that were dissolved in pet. with the aid of small amounts of acetone and diethyl ether. The solvents were then evaporated with a stream of nitrogen. Concentrations tested were 1.2, 0.40, 0.13% (3.0×10^{-5} , 1.0×10^{-5} , 3.2×10^{-6} mol/g) and also 0.044% (1.1×10^{-6} mol/g) for series 2.

The statistical evaluation of the animal experiments was performed using the Fisher exact test and the McNemar test.

Table 2. Sensitizing capacity of crystalline MPA

Test conc. MPA (%)		Exposed (n=15) ^{a)}	Controls (n=15)
7.2	48 h	0	2
	72 h	2	0
3.6	48 h	2	3
	72 h	3	1
1.2	48 h	2	3
	72 h	3	2
0.40	48 h	2	2
	72 h	1	0
0.12	48 h	1	0
	72 h	1	0
7.2 ^{b)}	48 h	3	3
	72 h	5 (<0.05) ^{c)}	0
pet.	48 h	0	0
	72 h	0	0

^{a)} Induced with crystalline MPA 1.2% in pet.

^{b)} MPA in amorphous form mechanically incorporated in pet.

^{c)} *p*-value (exposed/controls).

Table 3. Eliciting activity of crystalline and dissolved MPA

		No. positive reactions in exposed group ^{a)}		
Test conc. MPA (%)		Test series 1 ^{b)} MPA mechanically incorporated in pet.	Test series 2 ^{b)} MPA dissolved in pet.	<i>p</i> ^{c)} (series 1/ series 2)
1.2	48 h	7 (<0.05) ^{d)}	14 (<0.001) ^{d)}	<0.05
	72 h	13 (<0.001)	13 (<0.001)	
0.40	48 h	8 (<0.01)	14 (<0.001)	<0.05
	72 h	11 (<0.001)	15 (<0.001)	
0.13	48 h	2	10 (<0.001)	<0.05
	72 h	8 (<0.005)	13 (<0.001)	
0.044	48 h	nt	11 (<0.001)	
	72 h	nt	13 (<0.001)	
pet.	48 h	0	0	
	72 h	0	0	

^{a)} 15 animals induced with MPA 3.5% in FCA/H₂O emulsion intradermally.

^{b)} The animals were challenged with one series of crystalline MPA mechanically incorporated in pet. and another series with crystalline MPA dissolved in pet. and also with a vehicle control. The patches were randomly distributed on both flanks and read blindly at the same time.

^{c)} Statistical calculations of the response obtained when testing with MPA mechanically incorporated in pet. compared to the response from dissolved MPA (McNemar test).

^{d)} *p*-value (exposed/controls). Only one positive reaction was observed in the control group.

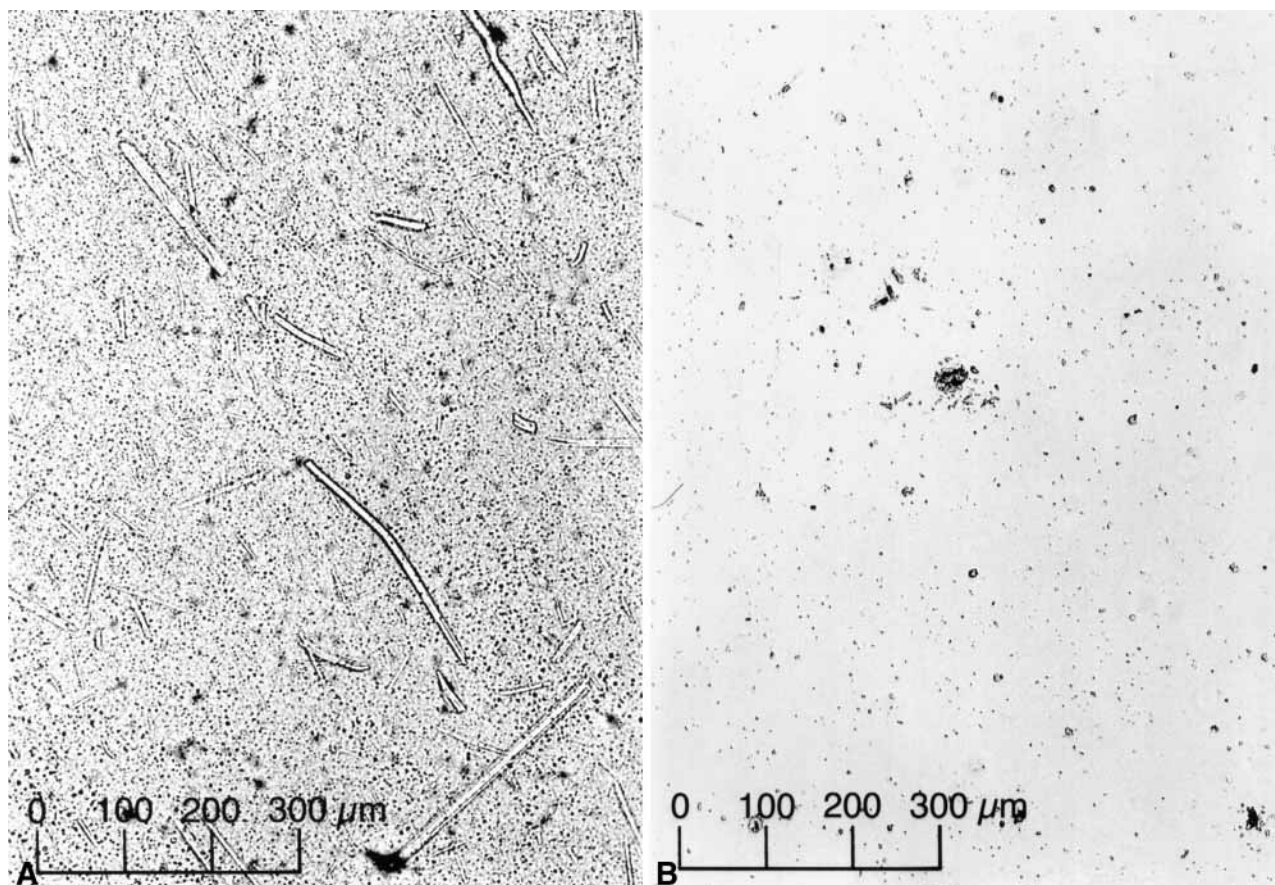


Fig. 2. (A) 1.2% MPA in pet. The crystals were ground finely and mechanically incorporated into pet. (B) 1.2% MPA in pet. The crystals were dissolved in ether and acetone, mixed with pet, followed by evaporation of the solvents.

Results

Dissolution of ^3H -MPA from pet. into synthetic sweat

The dissolution from the preparation in which MPA was mechanically incorporated into pet. was 2215 dpm per 10 ml of synthetic sweat. The dissolution from the dissolved MPA was 6556 dpm per 10 ml of synthetic sweat.

Sensitization and elicitation experiments in guinea pigs

Sensitizing capacity of crystalline MPA. In those animals that were epidermally induced with crystalline MPA, no significant response was observed to any of the concentrations of crystalline MPA. A significant response ($p < 0.05$) was observed at the 72-h reading to 7.2% amorphous MPA in pet. Some irritation was observed in the control animals (Table 2).

Eliciting capacity of crystalline and dissolved MPA. The animals were sensitized by the intrader-

mal induction. A significant response was observed for all test concentrations of MPA dissolved in pet. both at 48 and 72 h. Only 1 positive reaction was seen in the controls. A significant response was also observed for the serial dilution of crystalline MPA in pet. except for 0.13% at 48 h. The number of reactions was fewer for the serial dilution of crystalline MPA compared to the serial dilution of dissolved MPA. The difference was statistically significant for all concentrations at the 48 h reading (Table 3).

Discussion

A lower eliciting capacity was obtained from crystalline MPA compared to that obtained from dissolved MPA in guinea pigs intradermally induced with MPA. Using crystalline MPA for epidermal induction and challenge testing gave no significant response. This is in contrast to the significant sensitization that was obtained with amorphous MPA in a corresponding experiment (3).

To test the hypothesis that the difference in al-

lergenic activity observed could depend upon the physical form of MPA in the test preparation, we studied the release of MPA from 2 different preparations. About $3\times$ as much MPA was detected in synthetic sweat when MPA was dissolved in ether before mixing with pet. compared to when MPA was mechanically incorporated. MPA has a very low solubility in water. Therefore, we decided to use tritium-labelled material to determine the dissolution, rather than to use a chemical analysis that could be cumbersome with the very low levels of dissolved MPA in the synthetic sweat used as water phase. 2 preparations of MPA in pet. were photographed under the microscope (Fig. 2). The preparations look completely different. Needle-like crystals are easily distinguishable in the preparation where crystalline MPA was mechanically ground into pet. In the other preparation, where MPA was dissolved in ether before addition to pet., small aggregates are visible. These differences in physical appearance and dissolution rate are likely to explain the differences seen in the allergic response (Table 3), since the bio-availability should increase if MPA is dissolved in the vehicle (15, 16).

The results obtained in this study are in accordance with the results obtained in our previous patch test study on consecutive dermatitis patients (7). In that study, significantly more reactions were seen to crystalline MPA compared to amorphous MPA (Table 1). The amorphous form of MPA is likely to have a larger surface area than crystalline MPA and the molecules to be less ordered. Both these properties may give the amorphous MPA a higher dissolution rate, resulting in higher concentration in solution in the vehicle (15) and in greater bio-availability.

The solubility of a compound in a topically-applied vehicle affects the release rate to the skin in 2 opposing ways. A high solubility of a compound in a vehicle, i.e., a higher effective concentration, results in a high concentration gradient between the vehicle and the skin. This enhances the release rate from the vehicle. However, a higher solubility in the vehicle decreases the partition coefficient between the skin and the vehicle resulting in a lower release rate into the skin. The maximum skin bio-availability would therefore be when these 2 opposing mechanisms are balanced (17). In the case of MPA in amorphous form or the MPA dissolved into pet. with the aid of solvents, the higher portion of solubilized MPA in pet. results in a higher concentration gradient over the vehicle-skin barrier. The solubility of MPA in pet. seems, however, low enough to favour the partition from the vehicle into the skin.

An observation similar to ours was made by

Ostrenga et al. (18) for 0.05% fluocinonide creams with varying amounts of solubilizer (propylene glycol) in the cream base (carbowax, fatty alcohol and polyvinyl pyrrolidone). Higher release rates in vitro and a higher in vivo response (vasoconstriction) correlated with % of drug solubilized in the cream. Using 0.05% fluocinonide in pet. with varying amounts of solubilizer, a good correlation was observed between the concentration of solubilized drug and the in vivo response. As opposed to our observations, Hughes et al. (19) found no difference in skin absorption between substituted aromatic compounds in different physical forms, i.e., as solids, in ethanolic solutions, in aqueous pastes, or in aqueous suspensions. This may be an example of compounds for which the release rate from the topically applied preparations is of secondary importance in comparison to the rate-limiting percutaneous absorption step.

We have previously compared the response from 2 preparations of unmodified colophonium in pet. when patch tested in colophonium-sensitive persons (20). In one preparation, the colophony was mechanically incorporated. In the other preparation, colophony was dissolved in diethyl ether, pet. was added, and the diethyl ether was subsequently evaporated. Colophonium-sensitive patients reacted to the same extent to both preparations. However, since colophonium is an amorphous material, none of the preparations contained allergens in crystalline form (20). Furthermore, modified colophonium exists as amorphous solids and sometimes as highly viscous liquids. Thus, when handling colophonium or colophonium-containing products in industry or by consumers, the exposure will probably be to the non-crystalline forms of MPA. When screening dermatitis patients with crystalline MPA or other crystalline substances of low water solubility, cases of contact allergy could be overlooked.

At present, no MPA is commercially available for patch testing. However, since MPA is a strong allergen found in modified colophonium, we recommend the use of a preparation of MPA dissolved in pet. for patch testing of, e.g., cases with suspected allergic contact dermatitis from footwear and paper products.

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