

Stability of thiuram disulfides in patch test preparations and formation of asymmetric disulfides

O. BERGENDORFF AND C. HANSSON

Department of Dermatology, Lund University, University Hospital, Lund, Sweden

The thiuram mix used in patch testing originally contains 4 compounds. However, chemical analysis of the test preparation revealed that several new compounds are spontaneously formed during storage. The structures of these compounds have been determined and the rate of their formation has been studied in buffer solution at pH 7.4. After a few hours, a large amount of mixed disulfides are formed in solutions originally containing only symmetric disulfides. The impact on the test result of the formation of asymmetric disulfides has been investigated by testing on thiuram-sensitive volunteers with different preparations of mixed thiuram disulfides. In our study, the formation of new asymmetric thiuram disulfides from the original symmetric thiuram disulfides in the test preparation had no influence on the result of the patch testing. However, as the chemical analysis showed that the mix composition changes during the period when the preparations are used, and differs between suppliers, the question is raised as to whether it is acceptable to use test preparations with a composition that is different from that labelled on the protocol.

Key words: allergic contact dermatitis; high-performance liquid chromatography; HPLC; rubber chemicals; thiuram disulfides; patch testing technique. © Munksgaard, 2001.

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Contact dermatitis from thiurams is the most common reason for rubber allergy (1–3). Thiurams are added as accelerators in rubber processing, and unreacted thiurams remain in the finished rubber product. Hypersensitivity to thiurams is tested for by using the thiuram mix, which consists of 4 components: tetramethylthiuram monosulfide (TMTM), tetramethylthiuram disulfide (TMTD), tetraethylthiuram disulfide (TETD) and dipentamethylenethiuram disulfide (PTD) (Fig. 1). This mix is recommended as a screening test (4). The specific hypersensitivity may then be elucidated in a 2nd test series using a separate test for each individual thiuram.

In an ongoing study on the chemical reactivity and hapten activation of thiurams, we have experienced rapid reactions of thiuram disulfides with other disulfides and thiols. Our earlier findings concerning stability of the mercapto mix (5) justified investigation of the stability of the thiuram mix. In this paper, we present an HPLC method for analysis of all 4 components of the thiuram mix, together with 3 new compounds formed spontaneously within the mix, in a single run. This

method is now used for analysis of the thiuram mix and the stability of these compounds under biomimetic conditions. The analyses have shown that the thiuram mix components react with each other to give new compounds. This has previously been shown to occur in hydrophilic gels (6) as well as in solution (7, 8). The chemical reactions of thiurams in the test preparation result not only in the formation of new compounds with unknown allergenicity, but also in a lowering of the concentrations of the haptens we wish to test with. To investigate how these changes in the test material might influence the test result, we decided to perform patch tests on thiuram-sensitive volunteers using thiuram preparations of controlled composition.

Materials and Methods

TMTM, TMTD, TETD and PTD were obtained from Janssen Chimica. Acetonitrile and tetrahydrofuran (both HPLC grade) were obtained from Labscan Ltd., Dublin, Ireland. Thiuram mixes

were obtained from Chemotechnique Diagnostics, Sweden, and ALK Sverige AB, Sweden.

The mass spectra (EI ionization, direct inlet) were recorded with a Jeol SX102 (Tokyo, Japan) spectrometer at 70 eV.

Chromatographic analyses were performed by HPLC on a reversed-phase column (Supelcosil LC-18-DB, 150X4.6 mm, 3 μ m, Supelco Inc. Bellefonte PA, USA), eluted with acetonitrile: water: tetrahydrofuran (99: 99: 2) at a flow rate of 1 ml/min with a Waters 600 pump (Waters Chromatography division, Milford MA, USA) and monitored at 280 nm with a Spectromonitor III UV-detector (LDC/Milton Roy, Riviera Beach FL, USA).

Analysis of commercial thiuram mixes

A sample of Pharmacia TRUE TestTM thiuram mix patch was extracted in a glass vial containing 1 ml acetonitrile at room temperature in an ultrasonic bath for 15 s. The extract was immediately injected on the HPLC column.

As reference samples, 4 different solutions containing 1 mg/ml each of TMTM, TMTD, TETD and PTD were prepared. 5 μ l of each solution were put on separate filter paper discs and left to dry at room temperature for 30 min. The filter paper discs were put together in a glass vial, extracted and analysed in the same way as the TRUE TestTM patch.

A fresh sample of Chemotechnique 4 $\times$ 0.25% thiuram mix in petrolatum (pet.) (7.1 mg) was extracted in a Duall homogenizer with 2 ml of acetonitrile for 15 s. The extract was immediately injected on the HPLC column.

A 3-month-old Chemotechnique 4 $\times$ 0.25% thiuram mix in pet. was analysed in the same way as the fresh mix. This mix had been used at the clinic since it was bought and stored in a refrigerator when not used.

As reference samples, 25 mg each of Chemotech-

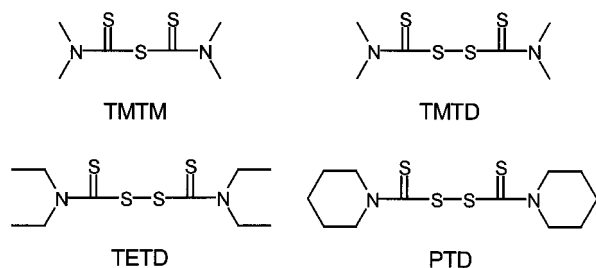
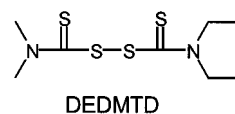


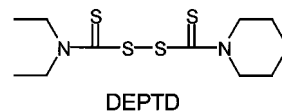
Fig. 1. Molecular structures of tetramethylthiuram monosulfide (TMTM), tetramethylthiuram disulfide (TMTD), tetraethylthiuram disulfide (TETD) and dipentamethylenethiuram disulfide (PTD).



DEDMTD



DMPTD



DEPTD

Fig. 2. Molecular structures of new disulfides formed in thiuram mix. Diethyldimethylthiuram disulfide (DEDMTD), dimethylpentamethylenethiuram disulfide (DMPTD) and diethylpentamethylenethiuram disulfide (DEPTD).

nique 1% TMTM, TMTD, TETD and PTD in pet. was mixed manually. Then 3.8 mg of this mix was extracted and analysed in the same way as the Chemotechnique 4 $\times$ 0.25% thiuram mix.

Synthesis of mixed disulfides for mass spectrometry

Diethyldimethylthiuram disulfide (DEDMTD, Fig. 2) was formed by mixing 5 μ mol TETD and 5 μ mol TMTD in 5 ml acetonitrile. The reaction was run in a test tube at room temperature for 24 h and monitored by HPLC. Diethylpentamethylenethiuram disulfide (DEPTD) and dimethylpentamethylenethiuram disulfide (DMPTD) were formed by the same method.

Study of the kinetics of mixed disulfide formation

3 solutions were made from 12 mg TMTD, 15 mg of TETD and 16 mg PTD each in 10 ml DMSO. 50 μ l of TMTD solution and 50 μ l of TETD solution were mixed with 9.0 ml buffer (0.05 M phosphate buffer, pH 7.4) and 1.0 ml DMSO containing diethyl phthalate as internal standard. DMSO was added to the buffer prior to the addition of thiurams in order to obtain a clear solution. Mixed disulfides from TMTD and PTD, and TETD and PTD were studied in the same way.

Preparation of test material

Patch tests of allergens in petrolatum were applied to the skin by using Finn Chambers[®] (Epitest Ltd Oy, Finland) mounted on Scanpor[®] (Norgesplaster A/S, Norway). The patches were removed after

2 days (D) and read at D3 and D7. The patch test protocol was designed to show whether the formation of asymmetric (mixed) thiuram disulfides in the patch test preparation would influence the result of the patch test. Therefore, the patients were tested either with preparations containing only symmetric disulfides or with preparations containing symmetric and asymmetric disulfides in equilibrium. Test nos. 1–8 contain 1 thiuram each in 1 or 0.25% pet. Test no. 9 contains all 4 thiurams, prepared immediately before application and represents a thiuram mix made as fresh as possible. HPLC analysis of this preparation showed that no asymmetric disulfides were formed prior to application. In test no. 10, 25 mg of each thiuram had been standing overnight in 4 ml of methyl tert-butyl ether before evaporation under a stream of nitrogen gas. The dry residue was then mixed with 10 g of pet. HPLC analysis showed that the reaction was in equilibrium, containing all 3 asymmetric disulfides besides the original 4 thiurams. Test

no. 11 was a fresh thiuram mix from Chemotechnique.

Results

Analysis of commercial thiuram mixes

The critical step in the investigation of mix preparations was to avoid the formation of artefacts during the extraction procedure. In the case of thiuram mixes, new compounds turned out to be produced easily in any solution containing 2 or more thiuram disulfides. Therefore, it was important to compare the result with adequate reference samples handled in a way that extraction-related reactions could be controlled. The time that was spent between addition of extracting solvent and

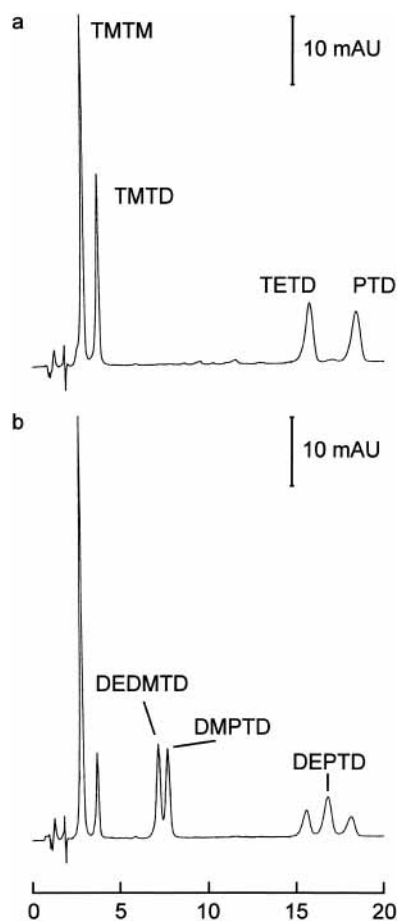


Fig. 3. HPLC chromatograms of thiuram mix patches. (a) Reference mix of the 4 components in the mix extracted from filter paper immediately before analysis. (b) Extract of a TRUE Test™ patch showing the original 4 thiurams and the 3 asymmetric thiurams formed.

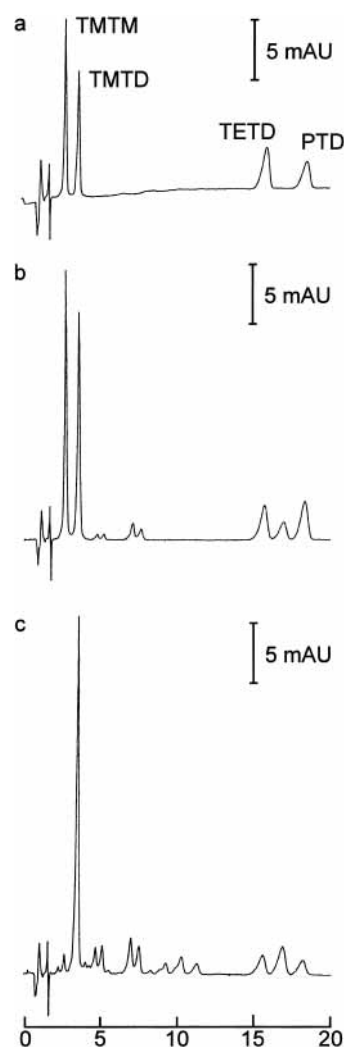


Fig. 4. HPLC chromatograms of petrolatum (pet.) preparations of thiuram mixes. (a) Reference mix of the 4 components in pet. The mix was prepared and extracted immediately before analysis. (b) Pet. preparation from Chemotechnique analysed 3 days after delivery. (c) Pet. preparation from Chemotechnique analysed after 3 months storage in refrigerator.

injection of the extract on the column must be minimized, but still long enough to give an effective extraction.

The TRUE Test™ patch consists of a polymeric plate coated with the allergen dissolved in a hydrophilic gel. To mimic the extraction of this material, filter paper discs were impregnated with thiurams and extracted in the same way as the TRUE Test™ patch (Fig. 3). HPLC analysis of these extracts shows that the test patch contains asymmetric thiuram disulfides (Fig. 3b). The absence of asymmetric disulfides in the reference chromatogram (Fig. 3a) indicates that the extraction technique does not produce any artefacts.

The pet. preparation could not be satisfactorily extracted in an ultrasonic bath because of the need

to mechanically work the pet. phase simultaneously with extraction. The use of a tissue grinder offered a fast and effective method of transferring thiurams in pet. to the organic solvent. Comparison of the chromatograms of samples and references in Fig. 4 shows that the extraction methods used do not produce mixed disulfide artefacts, due to possible interactions between thiurams in solution.

Besides the original thiurams, all test preparations contained new compounds. Comparison of the retention times of these compounds with products from synthesis of mixed disulfides confirmed the presence of DEDMTD, DEPTD and DMPTD in the mixes. In Fig. 4c, additional peaks are seen due to further reaction in the mix during storage. The structures of these peaks remain to be determined, but we suspect that they might be asymmetric monosulfides, produced from disulfides.

Synthesis of mixed disulfides

Analysis by HPLC of the thiuram mix preparations have shown 3 new compounds besides the original thiurams TMTM, TMTD, TETD and PTD. The new compounds were easily prepared in high concentrations by mixing equimolar solutions of the pure disulfides in acetonitrile or in buffer solutions at physiological pH. This procedure was used for each of the 3 pairs of disulfides (TMTD+TETD, TMTD+PTD and TETD+PDT). When the solutions were analysed by HPLC, a new peak was found between the original peaks (Fig. 5), and after

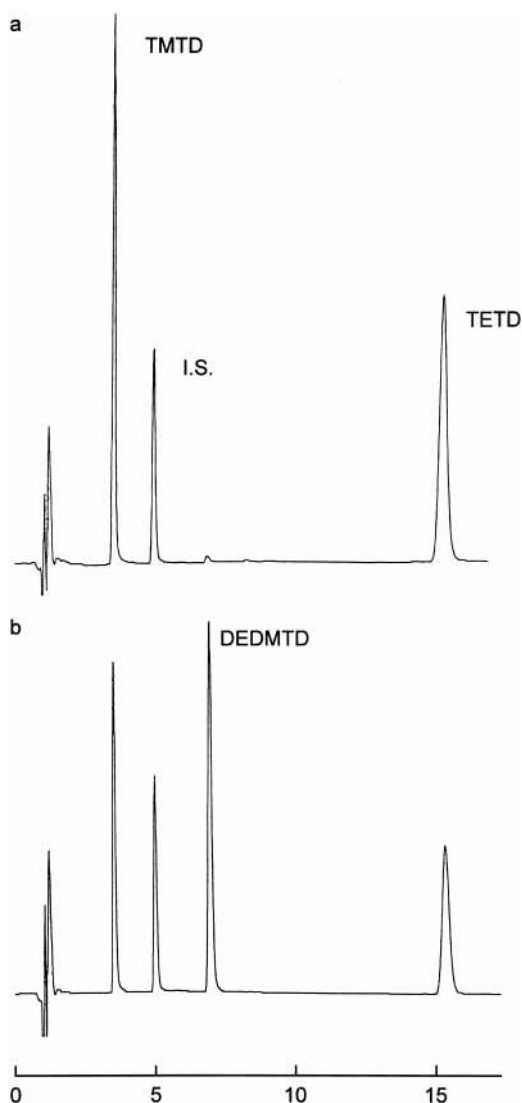


Fig. 5. HPLC analysis of the reaction between TMTD and TETD monitored at different times after mixture of reactants. (a) 1 min, (b) 7.5 h. I.S.=internal standard (diethyl phthalate).

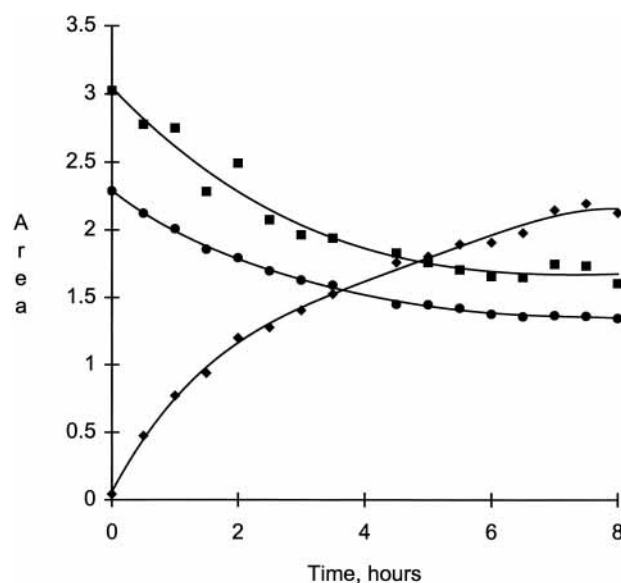


Fig. 6. The areas of TMTD: ■, TETD: ● and DEDMTD: ◆, relative internal standard in a chromatogram as a function of time after mixing TMTD and TETD.

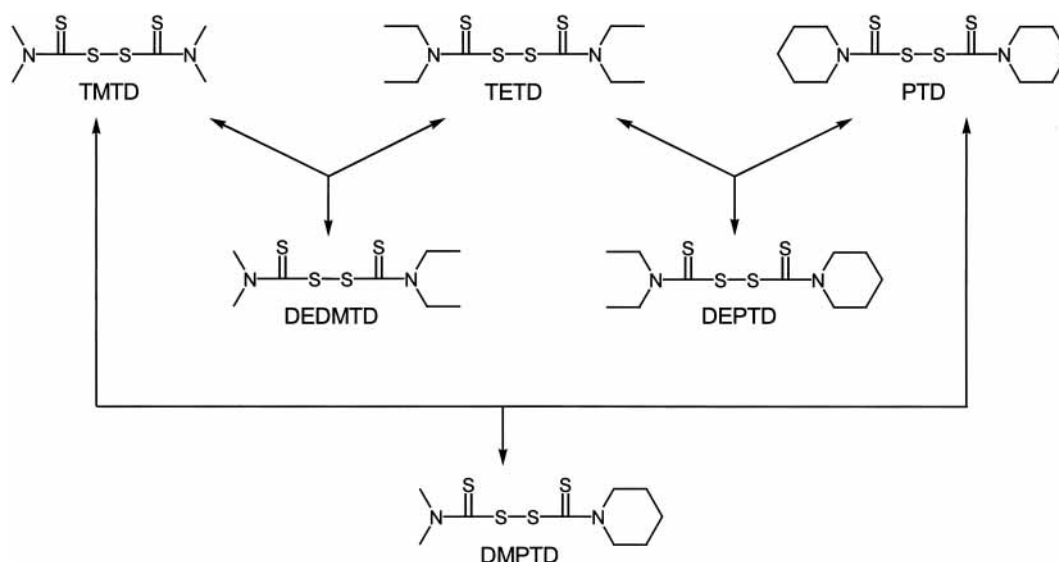


Fig. 7. A summary of different interconversions between thiuram disulfides.

6 h at room temperature, the reaction seemed to have reached an equilibrium (Fig. 6). At this point, each chromatogram showed 3 peaks: 2 peaks corresponding to the original disulfides, and 1 bigger peak with an intermediate retention time.

Attempts to isolate these new peaks failed, as a new equilibrium with the original disulfides was immediately established from the isolated product. Instead the new compounds were characterized by comparing mass spectra from the mixtures at equilibrium with mass spectra of the pure thiurams. The mass spectra of the mix between TMTD and TETD showed molecular ions from TMTD at m/z 240 and from TETD at m/z 296, and an additional signal at m/z 268 corresponding to a molecular ion from the mixed disulfide DEDMTD. Apart from known fragment ions from the thiurams (9), no additional peaks were detected. Mass spectra of the other 2 mixtures gave additional signals from DMPTD at m/z 280 and from DEPTD at m/z 308.

The chromatographic behaviour and mass spectral observations as well as the quantitative behaviour of the equilibrium between the disulfides are in accordance with the new compounds being asymmetric products of disulfide exchange.

Patch tests

The results of the patch tests are presented in Table 1. Only readings from D3 are shown, as essentially no new information was obtained at D7. The most important result of the patch test study can be found when comparing the 3 lowest rows in Table 1. Preparation no. 9 is an ideal thiuram mix, prepared immediately before application, when HPLC analyses showed only pure symmetric thiurams and no new asymmetric thiurams. Preparation no. 10 is the opposite, a completely equilibrated mixture of 4 symmetric thiurams and 3 newly formed asymmetric thiurams. Finally, no. 11 is a

Table 1. Patch test results

No.	Allergen(s)	Concentration % pet.	Patient no.									
			1	2	3	4	5	6	7	8	9	10
1	TMTM	1	+	++	+	+++	++	++	+++	++	—	—
2	TMTD	1	+	++	+++	+++	—	—	+	—	—	—
3	TETD	1	++	+++	++	++	—	—	—	++	+	—
4	PTD	1	+	+++	+	+++	—	—	—	—	—	—
5	TMTM	0.25	+	++	+	+++	++	++	++	+	—	—
6	TMTD	0.25	+	++	++	++	—	—	+	—	—	—
7	TETD	0.25	++	++	+++	+	—	—	—	+	—	+
8	PTD	0.25	+	+++	++	++	—	—	—	—	—	—
9	TMTM+TMTD+TETD+PTD	0.25×4	++	+++	++	+++	+	++	+++	+++	++	+
10	TMTM+TMTD+TETD+PTD	0.25×4	++	+++	++	++	+	+	+++	+++	+	+
11	TMTM+TMTD+TETD+PTD	0.25×4	++	+++	++	+++	+	++	+++	++	+	+

commercial thiuram mix from Chemotechnique also known to contain asymmetric thiurams. No significant difference can be seen between the readings of these tests.

Discussion

The diagnosis of allergic contact dermatitis due to thiuram sensitivity is usually based on patch testing with thiuram mix in the standard series. From chemical analysis of this mix we found several compounds which were not originally in the mix but formed spontaneously in the mix preparation. Chromatographic separations and spectroscopic analyses revealed the formation of asymmetric disulfides (Fig. 7). The reaction between the symmetric disulfides is so fast that it is impossible to test these in a mix without the formation of asymmetric disulfides. For the same reason, it is impossible to test with pure asymmetric disulfides because of the rapid formation of an equilibrium with their corresponding symmetric disulfides.

The relevance of asymmetric thiuram disulfides in rubber products is uncertain. We have been in contact with some rubber industries and in many products several different thiurams are added as accelerators. This makes it possible for mixed disulfides to be formed during rubber production. The HPLC method presented in this report is sensitive enough for analysis of mixed disulfides in products after vulcanization as well. Thus far, we have not been able to find any rubber product containing asymmetric thiuram disulfides. It is important to note that an extract from a rubber product must be analysed as fresh as possible. Otherwise, thiuram disulfides in the extract will react in the extraction solvent and form asymmetric disulfides.

The asymmetric thiuram disulfides presented in this work have evidently been present in thousands of standard test preparations over many years without any knowledge of their immunologic properties. However, we have no reason to believe that the formation of asymmetric thiuram disulfides in the test preparation or in the skin will have any effect on reactions at patch testing. How full antigens from thiurams and chemically related dithiocarbamates are formed is not fully understood. It is a well-known fact that thiuram disulfides react with protein thiols to produce a dithiocarbamoyl adduct (10). But these adducts are probably not stable enough to act as full antigens. Instead, synthetically made thiocarbamoyl adducts between serum albumin and dithiocarbamoyl chloride have served as models of a full antigen. Lymphocyte transformation studies on thiuram-sensitive patients showed significant responses to these ad-

ducts, and the authors conclude that the dialkylamine part of the molecule is the "relevant haptenic determinant" (11). This may explain our observation that asymmetric thiuram disulfides give the same patch test reactions as corresponding pairs of symmetric thiuram disulfides. Therefore, we believe that a full antigen formed after reaction with a thiuram disulfide or its metabolite will contain $1/2$ a molecule of the thiuram, i.e. a (di)thiocarbamate structure, and it is therefore not important if the hapten originates from a symmetric or an asymmetric thiuram disulfide.

The consequences of these chemical reactions in a test preparation are, firstly, that the concentration of the allergens that were meant to be tested with has decreased and, secondly, that new compounds of unknown relevance to the test are present. Although the number of patients tested in this study is limited, we have no evidence for any influence on the patch test results due to the formation of asymmetric thiurams. Another, and perhaps more important, question is whether it is acceptable to use test preparations with a different composition from that labelled on the protocol. Furthermore, this difference is not identical for test preparations from different suppliers and not stable during storage.

The instabilities found in the thiuram mix are similar to those that we have previously found in the mercapto mix (5). The ultimate question is whether, with this knowledge, we can justify the use of mix preparations.

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Address:

Ola Bergendorff
Department of Dermatology
University Hospital
SE-221 85 Lund
Sweden