

Extraction of haptens from solid products and their delivery to the skin, exemplified by dithiocarbamates from rubber gloves

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Allergic contact dermatitis is often caused by solid products such as rubber gloves. Patch testing with the product as is often gives negative results. Extraction of the haptens into an organic solvent is commonly performed to achieve a more correct investigation. The technique used for extraction of haptens from solid materials is only sporadically described. In this study, we investigated and optimized the yields of dithiocarbamates obtained by extraction from rubber gloves. The influence of solvent, extraction time and the procedures for extraction are evaluated. The delivery of zinc dibutylidithiocarbamate from the patch test preparation to the skin is determined.

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When patch testing with standard series or using any of the established special series, the concentrations of the haptens and the vehicles used are based on many years experience at several centres and evaluation by ICDRG. Correct concentrations have often been confirmed by tests with dilution series to obtain a moderate allergic response with minimal irritations.

Patch tests are applied as pure chemical substances in a suitable vehicle or as mixes of defined compositions. However, many contact allergies are achieved by contact with a product of solid material. These products often consist of a complex mixture of substances, mostly of unknown origin not accompanied by any declaration of contents. When testing with a solid product as is, a positive patch test sometimes appears. For some products, extraction of haptens into an organic solvent is a necessary procedure to obtain a positive test. For other patients, a positive test for a pure chemical is registered, but even though this chemical is known to be present in the solid material, no positive patch test is achieved by testing as is or as an extract. Despite the negative test results, there can be a clear correlation between contact with the product and deterioration of the eczema.

Rubber gloves are among the most common solid products giving rise to allergic contact dermatitis (1, 2). The gloves are usually produced far away from the clinic investigating the patient, and information about added rubber chemicals is often difficult to obtain. Furthermore, the chemicals added are sometimes chemically changed during the vulcanization procedure, giving other known allergenic compounds as well as new related compounds not yet investigated (3). We think that patch tests with rubber gloves as is or as an extract often give false-negative results. The instructions for the extraction procedure are limited (4, 5). No chemical evaluation of the technique has previously been presented. To investigate this issue, we analysed the concentrations of haptens in patch test preparations of extracts from rubber gloves and compared these with ordinary concentrations of haptens in the rubber test series recommended by ICDRG. We also analysed different ways to perform the extraction of allergenic products and optimized the essential parameters. By standardizing this extraction procedure of rubber gloves, the concentrations of the investigated haptens in the test preparation achieved could be optimized to be of about the same concentration as in the recommendation for the rubber series.

Materials and Methods

Analyses of a large series of rubber gloves have been performed at our laboratory. Throughout this study, we have used the same brand and batch of gloves which we have found contains intermediate concentrations of the rubber haptens zinc pentamethylenedithiocarbamate (ZPD) and zinc dibutyldithiocarbamate (ZDBC). These haptens have been found at glove analyses recently (2, 6). Acetone, methyl tert-butyl ether (MTBE) and acetonitrile (HPLC-grade) were obtained from Labscan Ltd, Dublin, Ireland. ZPD was obtained from Robinson Brothers, West Midlands, UK (technical grade), and ZDBC (>97%) was obtained from TCI Europe, Zwijndrecht, Belgium. Ultrasonication was performed in a Branson 50/60 Hz Ultrasonicator.

HPLC analyses

HPLC analyses were performed using a Waters 600 gradient master and a Rheodyne loop-injector. The mobile phase consisted of a mixture of aqueous ZnSO_4 (10^{-5} mol/l) in 50% acetonitrile (A) and 100% acetonitrile (B). The first 5 min of isocratic elution was performed using 100% A and then a linear gradient from A to B for 35 min giving 100% B followed by isocratic elution for 10 min with 100% B. All equipment in contact with the mobile phase after the injector was of non-metallic material, and the column used was an Alltima C_{18} , 4 μm , 150×4.6 mm, PEEK-lined (Alltech Associates, Deerfield, IL, USA) (7). The rubber accelerators were identified by their retention times in the HPLC chromatograms and by comparison with their UV spectra by using a diode array detector (Hewlett Packard Diode Array 1100-detector). The concentrations were determined by calculating peak area in the HPLC chromatogram registered at 280 nm. The detection limit was determined to about 10 $\mu\text{g/g}$ of zinc dithiocarbamate in the glove.

Extraction procedures

0.5 g (about 40 cm^2) of the glove was cut into small pieces, and solvent was added to completely cover the glove pieces. The amount of solvent was exactly 0.1 g rubber/ml. Extractions were then performed for 10 min in three different ways, all in duplicate:

- (i) by stirring using a 10 mm magnetic rod in a glass beaker of 50 mm diameter.
- (ii) by ultrasonication in the same glass beaker of 50 mm diameter.

- (iii) by shaking in a 10 ml glass test-tube sealed by a PEEK screw top.

After 10 min of extraction, the rubber was filtered off, and the extraction medium was evaporated to almost dryness by a stream of nitrogen. Evaporation time was about 10 min. The residue was dissolved in 1 ml acetonitrile and injected on to the HPLC system.

The effect of different extracting solvents was studied by using the shaking procedure. Distilled water, ethanol, MTBE and acetone were compared, and the extracts were analysed as described above.

Results

The extraction capacity of different solvents on rubber chemicals was evaluated, and acetone was found to give the best yield and was for that reason used in the subsequent experiments (Fig. 1).

By using acetone as solvent, three different techniques of extraction were evaluated: shaking, stirring and ultrasonication. The shaking procedure was found to be superior to the other 2 (Table 1). As regards ultrasonication, some differences were found depending on where in the sonication bath the vessel was placed.

We then investigated the extraction recovery at an repeated extraction time of 10 min when using shaking in acetone. Extraction of 0.5 g rubber glove cut in small pieces was performed by shaking using 5 ml acetone for 10 min. Then the acetone was pipetted off and analysed. Another 5 ml of acetone was added and extraction of the same rubber sample was performed for another 10 min. This 5 ml of acetone was then pipetted off and analysed, and a third portion of 5 ml of acetone was added for extraction for another

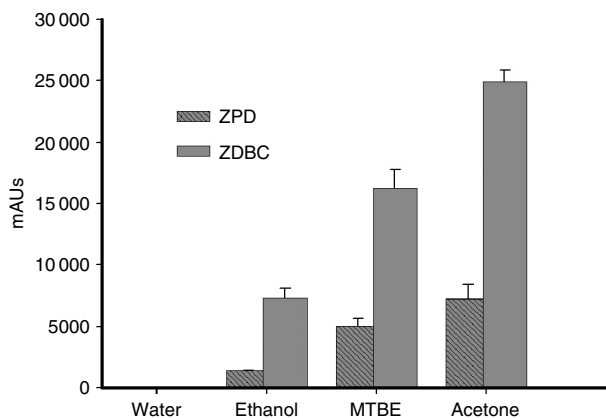


Fig. 1. Extraction capacity of different solvents for extraction of zinc pentamethylenedithiocarbamate (ZPD) and zinc dibutyldithiocarbamate (ZDBC) from rubber gloves. The values are given as mAU.

Table 1. Extraction yields of zinc pentamethylenedithiocarbamate (ZPD) and zinc dibutyldithiocarbamate (ZDBC) using different techniques for extraction of rubber gloves

	Shaking	Ultrasonication	Stirring
ZPD	14102 ± 1907	9354 ± 1569	8188 ± 1024
ZDBC	27597 ± 2841	18521 ± 4491	17943 ± 359

The values are given as mAU.

10 min, pipetted off and analysed (Fig. 2). We found that about 80% of the accelerators were extracted into the acetone phase already after the first 10 min. Small amounts of accelerators were found in the second portion and essentially nothing in the third portion. In a separate experiment, we have seen that with further extended extraction, more than 1 hr, we get a minor degradation of the haptens. Therefore, extraction times longer than 10 min are normally not justified. We also tried to decrease the amount of acetone at the extraction by using 0.25 g rubber/ml acetone instead of 0.10 g rubber/ml acetone. However, the extracted amounts of accelerators decreased from 80% to 65% during the first 10 min, and we found this procedure less suitable.

We then concluded that extraction of rubber gloves 0.1 g rubber/ml acetone by shaking for 10 min followed by evaporation using a stream of nitrogen to dryness and dissolving the residue in 50 µl acetone is an optimal way to obtain test extracts of rubber gloves. We therefore ran a triplicate of extraction using this procedure to determine its reproducibility. A high degree of reproducibility was found (Table 2).

By using this optimized procedure, dissolving all the 50 µl acetone in 20 mg petrolatum, we

Table 2. Investigation of the reproducibility of the proposed extraction procedure

	mg/extraction
Zinc pentamethylenedithiocarbamate	0.25 ± 0.02
Zinc dibutyldithiocarbamate	0.41 ± 0.03

The results are given as mean values and their standard deviations in a study of triplicates. The figures represent mg haptens achieved from 0.5 g rubber glove using the proposed procedure.

obtained a test preparation with about 0.25 mg ZPD and 0.40 mg ZDBC in 1 single-test preparation from the glove. This amount should be compared to the recommendations given by ICDRG. The amount of petrolatum applied from syringes by a skilful nurse is surprisingly constant between 16 mg and 20 mg. In the standardized test in the rubber series, zinc dithiocarbamates are recommended to be tested at 1% in petrolatum. The calculated amount of dithiocarbamate applied by using this recommendation is 0.18 mg, which is in the same range as the total amount achieved by the extraction procedure described above.

It is essential to know how much of the applied substance is transferred to the skin in patch testing. We thus studied the delivery of ZDBC to the skin at testing by using a paper disc or petrolatum as a vehicle in testing. Some extracts, after evaporation to dryness, were therefore dissolved in 50 µl acetone and then applied in two 25 µl portions to a filter paper disc (diameter 7 mm) which was placed in Finn Chambers[®] alumina cups at patch testing. Other identical 50 µl portions of extracts were dissolved drop by drop in 20 mg petrolatum applied to alumina cups and used when patch testing. This was performed in triplicate using rubber glove extracts prepared as described above. The 3 test preparations on filter paper discs were applied to the skin together with the 3 petrolatum dissolved extracts for 48 hr. Analyses of the samples on filter paper discs showed that 50% of ZDBC was left on the disc after patch testing. If the paper disc was completely dried before application on to the skin, a much lower delivery was registered. Corresponding experiments showed that the Finn Chamber[®] alumina cups used in petrolatum-based tests, and when extracted and analysed gave a residual 8% of ZDBC. Thus by using the optimized method described here on this type of gloves, a total 0.4 mg ZDBC is delivered to the skin when patch testing in petrolatum compared to 0.2 mg recommended by ICDRG.

Discussion

Epicutaneous testing is probably one of the most individually adapted test procedures performed

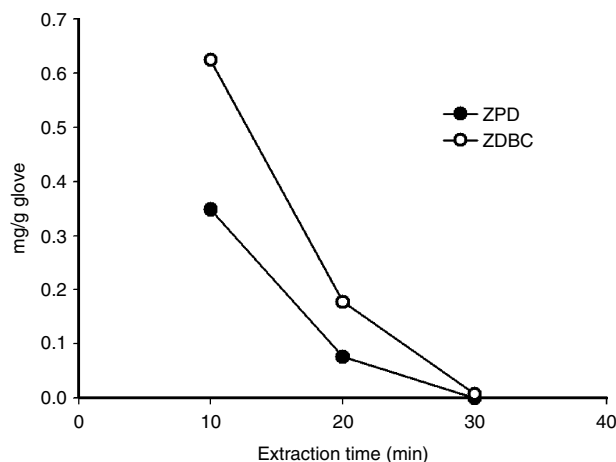


Fig. 2. Repeated extraction of zinc pentamethylenedithiocarbamate (ZPD) and zinc dibutyldithiocarbamate (ZDBC) from rubber gloves. 10 ml fresh acetone was added at 0 min, 10 min and 20 min, and each extraction was analysed after an extraction time of 10 min.

in health care. A high diversity of products is often presented by the patient. Some products such as cosmetics and chemicals used at home are labelled with the components. However, many products are solid and most often without any information on composition. These products give special difficulties because during testing as is they can give a sensitization of the patient due to an unexpectedly high concentration of the hapten. More often too low a delivery of haptens into the skin occurs and for that reason a false-negative test is obtained. Rubber gloves are one of the most common solid products giving allergic contact dermatitis (1, 8). It is often easy to suspect gloves as the reason for dermatitis due to the distribution of the skin reaction and to the correlation between the use of gloves and the deterioration of the dermatitis. In patch testing with rubber chemicals, thiurams are the most prevalent positive tests obtained, but nowadays thiurams are usually not found in analyses of rubber gloves. Instead, dithiocarbamate accelerators are commonly found by these analyses (2, 6). So it is often difficult to correlate glove dermatitis to a positive patch test in the rubber series by comparing the accelerators added at the glove production. Patch tests with solid rubber material as is or as an extract are therefore commonly performed. However, the concentrations of the rubber allergens in an extract are often unknown because no suitable method for analysis of dithiocarbamates has been available. Dithiocarbamates are chemically highly complexed with different metallic ions, and an analytic method must pay regard to this phenomenon (9). A recently developed HPLC method utilizing all PEEK-lined material has been used in this experiment (7, 10). This method gives essentially all allergenic rubber compounds in one single run. In the present study, we have used this method to evaluate the separate steps of the extraction procedure in obtaining test preparations from solid rubber products.

Acetone is an easily volatile, non-toxic solvent with excellent properties for extraction of dithiocarbamates from rubber gloves. We reported MTBE to be a better extraction solvent than acetone for extraction of 2-mercaptobenzothiazole (MBT) but found no difference for other mercaptobenzothiazoles derivatives (11). The rubber product extracted in that work was however much thicker than a rubber glove. The lipophilicity of MTBE can be of importance for penetration into the thicker rubber material. Although there have been reports on the reactions between acetone and thiurams and mercaptobenzothiazoles, we found these side-reactions

to be of importance only when high concentrations of haptens are present (11, 12). In a recent paper, Nakamura et al. (13) used a mixture of acetone and chloroform for extraction of MBT derivatives. However, this is a labourious technique using toxic chloroform and for that reason not suitable for use in routine patient work.

Comparison between different extraction techniques showed shaking to be superior to other tested techniques. Shaking gives possibilities for extraction of several samples simultaneously. Stirring or sonication gives only about half the amount of the haptens at extraction. A yield a few per cent higher can be achieved by optimizing the position of the vessel in the sonication bath.

The analyses presented in this paper show that evaporation almost to dryness of the extraction medium is essential to obtain high enough concentrations of the haptens. There have been proposals to use a hair fan with or without heating for this purpose. However most volatile organic solvents are highly ignitable in contact with hot material present in the fan, and for that reason, electrical apparatus should not be used. The evaporation of acetone by a stream of nitrogen is fast and without any risk of explosion or fire. Furthermore, the nitrogen atmosphere prevents oxidation during this step when extracting oxidizable haptens (14).

In previous studies, we have shown the instabilities of thiurams and mercaptobenzothiazoles in solutions (15, 16). The procedure described here did not indicate any reaction between the two dithiocarbamates investigated. If, however, several thiurams and/or mercaptobenzothiazoles are handled together in a solution, the risk of formation of new compounds during extraction is obvious. The extraction procedure described here is optimized for extraction of the rubber haptens studied. However, the extraction will also transfer other lipophilic compounds into the extract. Thus, there can be other allergens as well as other irritants present in the patch preparation, and extract test reactions diverging from testing with pure haptens may occur.

The main question in epicutaneous testing is the amount of hapten delivered into the skin in a standardized area. Our study shows that the optimized extraction procedure described here gives adequate amounts of dithiocarbamates in the test preparation from gloves with intermediate concentrations of dithiocarbamate accelerators. However, we also found that the delivery from paper filter disc into the skin is too low for dithiocarbamates. This observation is probably one reason for false-negative results when testing extracts from rubber gloves on paper filter discs.

The use of petrolatum as a vehicle is an acceptable alternative for the haptens studied in this work. Other haptens have other properties, and the delivery systems for these haptens have to be evaluated.

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