

Fragrance patch tests prepared in advance may give false-negative reactions

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Summary

Background. Several of the ingredients in fragrance mix I (FM I) have been shown to evaporate from petrolatum preparations applied in test chambers to an extent that can be suspected to affect the patch test result.

Objectives. To compare the reactivity towards FM I and fragrance mix II (FM II) when they are applied in test chambers in advance and immediately prior to the patch test occasion.

Methods. Seven hundred and ninety-five consecutive patients were simultaneously patch tested with duplicate samples of FM I and FM II. One sample was applied in the test chamber 6 days in advance (6D sample), and the other sample was applied immediately before the patients were patch tested (fresh sample).

Results. Twenty-two (2.8%) patients reacted exclusively to the fresh sample of FM I, 6 (0.7%) reacted exclusively to the 6D sample, and 22 (2.8%) reacted to both samples. The corresponding numbers for FM II were 9 (1.1%) for the fresh sample, 6 (0.7%) for the 6D sample and 12 (1.5%) for both samples.

Conclusions. There was a statistically significant difference between the numbers of patients reacting to the fresh and 6D samples of FM I. No corresponding difference was observed for FM II. This can probably be explained by differences in volatilities between the ingredients of FM I and FM II.

Key words: fragrance mix; preparation time; stability; volatility.

It has recently been recognized that several of the fragrance allergens included in fragrance mix I (FM I) evaporate from petrolatum preparations applied in test chambers to an extent that can be suspected to affect the outcome of the patch test (1, 2). Owing to the logistics in the clinical setting, it is sometimes necessary to load the test chambers with pet. preparations several hours or

even days before the test patches are applied to the back of the patient. The present study was performed in order to investigate whether this procedure leads to cases of missed diagnosis of fragrance contact allergy because of a decreased dose of allergens in patch tests of FM I and fragrance mix II (FM II) prepared in advance. The study was performed as a part of a larger study in which the relationships between reactions to the fragrance mixes, their constituents and other fragrance-related allergens in the baseline series were investigated (3). These findings will be published separately.

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Materials and Methods

Preparations of the FM I ingredients cinnamal, cinnamyl alcohol (Bedoukian, Danbury, CT, USA), hydroxycitronellal, eugenol (Firmenich Inc., Plainsboro, NJ,

Table 1. Concentrations of fragrance mix I (FM I) and fragrance mix II (FM II) ingredients in the mixes and in individual petrolatum preparations, and the vapour pressures of the pure substances

Ingredient	CAS no.	Vapour pressure (Pa at 25°C)	Concentration in mix (% wt/wt)	Concentration in individual preparation (% wt/wt)
FM I ingredients				
Amyl cinnamal	122-40-7	0.31	1.0	2.0
Cinnamal	104-55-2	3.6	1.0	1.0
Cinnamyl alcohol	104-54-1	2.4	1.0	2.0
Eugenol	97-53-0	1.3	1.0	2.0
<i>Evernia prunastri</i>	90028-68-5	—	1.0	2.0
Geraniol	106-24-1	1.7	1.0	2.0
Hydroxycitronellal	107-75-5	0.43	1.0	2.0
Isoeugenol	97-54-1	0.69	1.0	2.0
FM II ingredients				
Citral	5392-40-5	9.5	1.0	2.0
Citronellol	106-22-9	2.4	0.5	1.0
Coumarin	91-64-5	0.17	2.5	5.0
Farnesol	4602-84-0	0.049	2.5	5.0
Hexyl cinnamal	101-86-0	0.093	5.0	10.0
Hydroxyisohexyl 3-cyclohexene carboxaldehyde	31906-04-4	0.0039	2.5	5.0

USA), amyl cinnamal, geraniol, isoeugenol (International Flavors & Fragrances, Union Beach, NJ, USA) and *Evernia prunastri* (oak moss absolute; Robertet, Grasse, France) were prepared in pet. (Snow White Quality E; Apoteket Produktion & Laboratorier, Göteborg, Sweden) at our department at the concentrations given in Table 1. FM I consisting of the eight above-mentioned substances at a concentration of 1.0% (wt/wt) each was prepared in pet. together with 5.0% of the emulsifying agent sorbitan sesquiolate by Chemotechnique Diagnostics (Vellinge, Sweden). Similarly, the individual pet. preparations of the FM II ingredients citral, hexyl cinnamal (Firmenich Inc.), citronellol (Bedoukian), farnesol (Symrise GmbH & Co. KG, Holzminden, Germany), coumarin (Rhodia Opérations, Aubervilliers, France) and hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (International Flavors & Fragrances) were prepared at our department at the concentrations given in Table 1. FM II containing each of the six above-mentioned substances at concentrations corresponding to 50% of the concentrations used in the individual preparations was prepared in pet. by Chemotechnique Diagnostics. Substances of the same batches were used both in the individual preparations and in the fragrance mixes.

Between April 2009 and December 2010, 795 consecutive dermatitis patients (507 females; 288 males; mean age 47 years; range 16–88 years) were simultaneously

patch tested with duplicate samples of FM I and FM II and with preparations of the individual ingredients of the mixes. For each fragrance mix, one sample was applied to the test chamber (8-mm Finn Chambers®; Smart Practice, Phoenix, AZ, USA) 6 days in advance (6D sample), and the other sample (fresh sample) was applied to the test chamber immediately before the test chambers were applied to the patient's back. The pre-loaded test chambers were stored on trays in plastic bags. The plastic bags containing the trays were stored in a cupboard at room temperature. The separate ingredients of the mixes were patch tested after being freshly applied to the test chambers.

Twenty milligrams of each preparation was applied in the test chambers (4). The test chambers were removed by the patients after 48 hr, and the readings were performed by a dermatologist on D3 or D4 and on D7. The tests were scored according to the ICDRG criteria (5).

Statistics

McNemar's test (two-sided) was used to compare the numbers of patients reacting to the fresh and to the 6D preparations of FM I and FM II. McNemar's test was also used to compare the numbers of patients with stronger reactions to the fresh sample than to the 6D sample and vice versa. In these calculations, all doubtful reactions were regarded as negative. Differences were considered to be significant at $p < 0.05$.

Results

The results of the patch tests with fresh and 6D samples of FM I and FM II are shown in Tables 2 and 3. There was a significant difference between the number of patients reacting to the fresh sample of FM I and the number reacting to the 6D sample of FM I ($p = 0.0037$). Twenty-two (2.8%) patients reacted exclusively to the fresh sample, 6 (0.7%) reacted exclusively to the 6D sample, and 22 (2.8%) reacted to both samples. Five of 22 patients reacting exclusively to the fresh sample had doubtful reactions to the 6D sample. Two of 6 patients reacting exclusively to the 6D sample had doubtful reactions to the fresh sample. A significant difference was also observed when the strengths of the reactions to the fresh and 6D samples were compared ($p < 0.001$). Twenty-five patients showed stronger reactions to the fresh sample than to the 6D sample, 6 patients showed stronger reactions to the 6D sample than to the fresh sample, and 19 showed positive reactions of the same strength to both samples.

No statistically significant difference was found between the number of patients reacting to the fresh

Table 2. Positive reactions to samples of fragrance mix I (FM I) and fragrance mix II (FM II) applied in the test chambers 6 days in advance (6D) and immediately prior to the patch testing (fresh)

Test preparation	Strength of reaction	Number (%) of reactions			
		Only fresh	Only 6D	Fresh and 6D	
				Fresh	6D
FM I	+	18 (2.3)	6 (0.8)	7 (0.9)	10 (1.3)
	++	2 (0.3)	0	11 (1.4)	8 (1.0)
	+++	2 (0.3)	0	4 (0.5)	4 (0.5)
	Total	22 (2.8)	6 (0.8)	22 (2.8)	
FM II	+	9 (1.1)	6 (0.8)	5 (0.6)	5 (0.6)
	++	0	0	5 (0.6)	5 (0.6)
	+++	0	0	2 (0.3)	2 (0.3)
	Total	9 (1.1)	6 (0.8)	12 (1.5)	

sample of FM II and the number reacting to the 6D sample of FM II ($p > 0.3$). Nine (1.1%) patients reacted exclusively to the fresh sample, 6 (0.7%) reacted exclusively to the 6D sample, and 12 (1.5%) reacted to both samples. No significant difference was observed when the strengths of the reactions to the fresh and 6D samples were compared ($p > 0.3$). All patients reacting exclusively either to the fresh or to the 6D sample showed + reactions, and all patients reacting to both samples showed the same degree of reactivity to both samples. Two of 9 patients reacting exclusively to the fresh sample showed doubtful reactions to the 6D sample, and 1 of 6 patients reacting exclusively to the 6D sample showed a doubtful reaction to the fresh sample.

Thirty-eight patients reacted to at least one of the individual ingredients of FM I (Table 4). Twenty-nine of these patients also showed reactions to the fresh and/or 6D sample of FM I. Among the 22 patients reacting exclusively to the fresh sample of FM I, 8 reactions to the ingredients of FM I were observed in 6 patients. Four reactions were observed in 3 patients reacting exclusively to the 6D sample, and 34 reactions were observed in 20 patients reacting to both samples. Nine reactions to the ingredients of FM I were observed in 9 patients who showed negative (5 patients) or doubtful reactions (4 patients) to the fresh and/or the 6D sample of FM I.

Twenty-six patients reacted to at least one of the individual ingredients of FM II (Table 5). Seventeen of these patients showed simultaneous reactions to the fresh and/or 6D sample of FM II. Among the 9 patients reacting exclusively to the fresh sample, seven reactions to the ingredients of FM II were observed in 5 patients. Two reactions were observed in 2 patients reacting exclusively to the 6D sample, and 11 reactions were observed in 10 patients reacting to both samples. Nine reactions to the ingredients of FM II were observed in 9 patients

Table 3. Strength of patch test reactions in 65 subjects with positive reactions to fragrance mix I (FM I) and/or fragrance mix II (FM II) samples applied in the test chambers 6 days in advance (6D) and/or immediately prior to the patch test occasion (fresh)

Case no.	FM I		FM II	
	Fresh	6D	Fresh	6D
1–3	–	–	–	+
4–6	–	–	+	–
7 and 8	–	–	+	(+)
9 and 10	–	–	+	+
11	–	–	++	++
12	–	–	+++	+++
13	–	(+)	(+)	+
14	–	(+)	+	+
15–18	–	+	–	–
19	(+)	–	+	–
20 and 21	(+)	+	–	–
22–32	+	–	–	–
33 and 34	+	–	+	–
35	+	–	+	+
36–39	+	(+)	–	–
40–45	+	+	–	–
46	+	+	+	+
47	++	–	–	–
48	++	–	+	–
49 and 50	++	+	–	–
51	++	+	++	++
52–55	++	++	–	–
56 and 57	++	++	–	+
58	++	++	++	++
59	++	++	+++	+++
60	+++	–	–	–
61	+++	(+)	(+)	(+)
62 and 63	+++	+++	–	–
64 and 65	+++	+++	++	++

who showed negative (4 patients) or doubtful reactions (5 patients) to the fresh and/or the 6D sample of FM II.

Discussion

Patch testing with the fragrance mixes can be associated with problems of reproducibility and interpretation. It has been reported that a large proportion of patients who react to the fragrance mixes do not react to the ingredients of the mixes when they are tested separately (6–9). It has been hypothesized that the reason for this may be false-positive reactions to the mixes (7), additive effects of simultaneous reactions to the mix ingredients, increased skin penetration resulting from the irritancy of other constituents of the mixes, including the emulsifier sorbitan sesquileate in FM I (8), or the formation of new allergens in the mixes (9). Furthermore, it has been shown that a considerable proportion of fragrance-allergic patients

Table 4. Numbers of positive reactions to ingredients of fragrance mix I (FM I) and their distribution with regard to reactions to FM I samples applied in the test chambers 6 days in advance (6D) and immediately prior to the patch testing (fresh)

Test preparation	FM I-positive			FM I-negative
	Only fresh	Only 6D	Fresh and 6D	
Amyl cinnamal	0	0	0	0
Cinnamal	1	1	6	3
Cinnamyl alcohol	1	1	5	0
Eugenol	0	0	4	1
<i>Evernia prunastri</i>	3	1	9	1
Geraniol	1	0	0	1
Hydroxycitronellal	0	0	5	1
Isoeugenol	2	1	5	2
Total number of reactions (number of patients)	8 (6)	4 (3)	34 (20)	9 (9)

Table 5. Numbers of positive reactions to ingredients of fragrance mix II (FM II) and their distribution with regard to reactions to FM II samples applied in the test chambers 6 days in advance (6D) and immediately prior to the patch testing (fresh)

Test preparation	FM II-positive			FM II-negative
	Only fresh	Only 6D	Fresh and 6D	
Citral	3	0	3	5
Citronellol	1	0	0	0
Coumarin	0	0	1	1
Farnesol	3	1	1	1
Hexyl cinnamal	0	0	1	0
Hydroxyisohexyl 3-cyclohexene carboxaldehyde	0	1	6	2
Total number of reactions (number of patients)	7 (5)	2 (2)	11 (10)	9 (9)

react to the individual ingredients of the fragrance mixes but not to the mixes themselves, when the ingredients and the mixes have been simultaneously patch tested (10). Some fragrance compounds auto-oxidize upon air exposure into new possible allergens, as is seen, for example, for geraniol (11), cinnamyl alcohol (12), and citronellol (13). Recently, several of the ingredients in FM I have been shown to evaporate from pet. patch test preparations applied in test chambers to an extent that can be suspected to affect the outcome of the patch test (1, 2). The present study was performed in order to evaluate the clinical implications of these findings. We wanted to compare the reactivity between fresh samples and samples applied

to the test chambers a couple of days in advance, in which a substantial decrease in concentration of the fragrance compounds might be expected. Our patch test clinic usually operates on Mondays and Thursdays, and for practical reasons we chose to prepare the samples 6 days in advance. The tests used on a Monday were prepared on Tuesday in the previous week, and the tests used on a Thursday were prepared on Friday in the previous week.

A statistically significant difference was observed between the number of patients reacting to the fresh sample of FM I and the number reacting to the 6D sample of FM I. Among the 22 patients reacting exclusively to the fresh sample, in total 8 reactions to the components of FM I were observed in 6 patients. The number of reactions for each component was too small for any extensive conclusions to be drawn. Among the ingredients in FM I, cinnamal has been found to evaporate most quickly from pet. preparations. The concentration of cinnamal in samples of FM I from the same batch as used in the present study was found to be ~2% of the initial concentration when the samples were stored in Finn Chambers® at room temperature for 6 days (1). It could be expected that patients reacting (weakly) to cinnamal to a larger extent would react exclusively to the fresh sample, but only one reaction to cinnamal was observed in the group reacting exclusively to the fresh sample. Six of 7 patients who reacted simultaneously to cinnamal and the fresh sample of FM I showed ++ or +++ reactions to the latter. Three of 7 patients who reacted simultaneously to cinnamal and the 6D sample of FM I showed ++ or +++ reactions to the latter. Although the concentration of cinnamal is considerably lower in the 6D sample than in the fresh sample, it may still be sufficient to elicit positive reactions, especially in patients with a strong allergy (14). Furthermore, 5 of 7 patients who reacted simultaneously to cinnamal and the fresh sample of FM I also reacted to cinnamyl alcohol. Three of these patients also reacted to at least one other FM I ingredient.

Interestingly, we found no significant difference between the number of patients reacting to the fresh sample of FM II and the number reacting to the 6D sample of FM II. With the exception of HICC, the stabilities of pet. preparations of FM II ingredients applied in test chambers are not known. HICC has been found to evaporate to only a minor extent from pet. preparations applied in test chambers stored at room temperature (15).

A measure of the volatility of a pure substance is its vapour pressure, that is, the pressure exerted by a vapour when the vapour is in equilibrium with its liquid or solid phase. The higher the vapour pressure, the more volatile the substance. The rates of evaporation of both fragrance

compounds and acrylates/methacrylates from pet. preparations appear to be related to the vapour pressures of the pure substances (1, 15, 16).

With the exception of citral and citronellol, all of the substances in FM II have vapour pressures lower than that of amyl cinnamal, which has been found to be the most stable of the ingredients in FM I when analysed as individual pet. preparations (1). The low vapour pressures of several of the substances in FM II may explain why the reactivities to the fresh and 6D samples were essentially the same. HICC has been shown to be the most common sensitizer of the FM II ingredients in Swedish dermatitis patients (17). This was the case also in the present study. The large proportion of positive reactions to HICC among the FM II-allergic patients may partly explain why a significant difference in the number of reactions to the fresh and 6D samples of FM II was not observed. Citral has the highest vapour pressure of the FM II ingredients. Among 6 patients reacting simultaneously to citral and the fresh sample of FM II, 3 reacted exclusively to the fresh sample and not to the 6D sample. This may be attributable to the high vapour pressure of citral, which results in a lowering of the concentration of citral in the 6D preparation of FM II to an extent that results in negative reactions. Further chemical investigations on the stability of citral would be of interest, especially as the vapour pressure of citral is higher than that of cinnamal.

A limitation of the study is that we only compared the reactivity to the fresh fragrance mix samples with the reactivity to samples that had been stored in test chambers for 6 days. At least with regard to FM I, it would be of interest to also examine the reactivity to samples applied to the test chambers a couple of hours or up to a day prior to the patch test occasion, which would correspond to a more realistic situation in a patch test clinic. However, during preparation for larger studies or when pre-loaded test chambers are sent by mail, it is not unlikely that the pet. preparations will be applied to the test chambers

several days in advance. In these situations, test chambers with a built-in protecting cover, such as the IQ chamber™ (Chemotechnique Diagnostics), are usually preferred. In the present study, we used only Finn Chambers®, but from our previous study we know that the stability of cinnamal pet. preparations is only slightly better when they are stored in IQ chambers™ than when they are stored in Finn Chambers® (1). When studying the stability of selected fragrances and acrylates/methacrylates, Mose et al. found the van der Bend™ transport container to be the best alternative for storing or transporting single doses of pet. preparations of these volatile allergens (2).

To conclude, we have observed that the previously observed evaporation of ingredients in FM I from pet. preparations is of clinical importance, and that there were significantly more reactions when patch testing was performed with a fresh sample than when it was performed with a sample applied to the test chamber 6 days in advance. However, we were unable to show that this was caused by the evaporation of any particular constituent of FM I. The low number of positive reactions to the 6D sample of FM I can probably be explained by the total decreases in concentrations of the ingredients in the mix. A corresponding difference between the fresh sample and the sample applied in advance was not observed for FM II, probably because of the low volatility of several of the pure ingredients of the mix. However, knowledge is lacking about the stability of most of the ingredients of FM II when present in pet. preparations. It can therefore not be ruled out that single substances of the mix evaporate to an extent that may affect the outcome of the patch test.

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