

The ferric chloride spot test for the evaluation and standardization of emulsions of allergens

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A chemical spot test, the first of its kind, with ferric chloride has been developed as a safety test and for standardizing and monitoring water-in-oil emulsions, particularly of allergens. The test is simple, rapid, and reliable. It has eliminated the need for microscopy, conductivity, or re-emulsification before injection. With the use of this test, uniform emulsions can be provided for evaluating the therapeutic results of the repository technique employed in the treatment of pollinosis under controlled conditions.

In the search for improved therapy for allergic patients, the repository or adjuvant procedure employing water-in-oil emulsions of allergens continues to be investigated by some allergists. Of utmost concern is the possibility of constitutional reactions arising from inadequate emulsions containing unemulsified allergens.

A number of methods for evaluating emulsions are available which are either inadequate, such as the "feel" of the emulsion or the drop-on-water test, or are too complex or cumbersome to perform, such as microscopy, light scattering, and conductance. For these reasons the writer developed a simple chemical test, the ferric chloride spot test, which was described in 1961.¹ The application of this test during the past five years to a variety of emulsions injected into animals and man has demonstrated its reliability as an indicator of degree of emulsification and as a method of standardization of these emulsions. It has eliminated

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the need for microscopy and re-emulsification of emulsions of allergens used in the treatment of patients.²

It is the purpose of the present communication to explain more fully the mechanism of the test and to describe its uses and limitations.

THE FERRIC CHLORIDE SPOT TEST

Description of the test

The ferric chloride spot test is based on the Prussian blue reaction.³ A drop of the emulsion into which ferric chloride has been incorporated is placed on filter paper, previously saturated with potassium ferrocyanide, and observed for color reactions. If a blue ring forms immediately about the drop, it is considered positive and indicates incomplete emulsification. Conversely, if no reaction occurs it is reported as negative and indicates a "complete" emulsion, i.e., the absence of enough free (unemulsified) aqueous phase to be detected chemically by the ferrocyanide. The deeper the color and the wider the ring, the poorer the emulsion.

The theory behind the chemical spot test for water-in-oil emulsions (W/O) is simple. Complete W/O emulsions have no unemulsified aqueous phase and consist of dispersed water droplets of about 0.5 to 1.5 μ in size, each of which is surrounded by oil. The emulsion then has the properties of the oil, as though water were not present at all. It will float on water, will show no conductivity, and any chemical present in the aqueous phase cannot be detected. On the other hand, incomplete emulsions in which ferric chloride is incorporated have an unemulsified aqueous phase so that they are disrupted when floating on water, will conduct a current, and will react chemically by spot testing with potassium ferrocyanide.

The test gives its sharpest reaction when the saturated filter paper is dried. Although reactions also occur when the paper is wet, they are less accurate. In addition to the blue ring produced about the incomplete emulsion drop as described above, a light blue spot equal to the size of the drop (which is visible when the paper is reversed) is produced at the point of contact of the drop with the pretreated filter paper. The blue spot is perhaps a more sensitive indicator than the blue ring, since it is noted just before the blue ring appears during demulsification of a complete emulsion. This may be the first evidence of the Prussian blue reaction, but it is not as sharp or distinct as the appearance of the blue ring and should not be relied upon in performing the test.

The reading of the test must be made immediately after contact of the drop with the filter paper, since if sufficient time were allowed to elapse, the filter paper would draw the liquid out of the emulsion, releasing ferric chloride to produce the reaction. Within a few hours, syneresis of the emulsion becomes complete and a solid blue spot remains on the paper. With some emulsions, for example, those made with extracts of plantain, the blue may be tinged with brown.

Empirically, it was determined that one drop (0.05 ml.) of a 10 per cent solution of ferric chloride, added for each milliliter of allergen extract, served best in eliciting the reaction. It was rarely necessary to increase this concentration, for example, when highly concentrated plantain extract was used.

When ferric chloride is added to extracts of such allergens as house dust, pollens, and molds, it becomes quickly apparent that some reaction is taking place. Depending upon the type of allergen and the concentration employed, diverse color reactions ensue, and various degrees of flocculation may be noted. The nature of these reactions has not yet been determined.

Since these reactions may remove ferric chloride from solution, it is essential that a spot test be performed after its addition to the extract prior to emulsification. Failure to elicit the Prussian blue reaction indicates the need for additional ferric chloride (another drop per milliliter of extract) before emulsification is undertaken. There must obviously be an excess of Fe^{+++} ions in the aqueous phase (as elicited by the spot test shown in Fig. 1) before emulsification, or there will not be enough to provoke the test after emulsification, even in the poorest of emulsions.

A 0.02N solution of $\text{K}_4\text{Fe}(\text{CN})_6$ was used for impregnating the filter paper, either by submersion of the paper or by the use of a dropper, with the

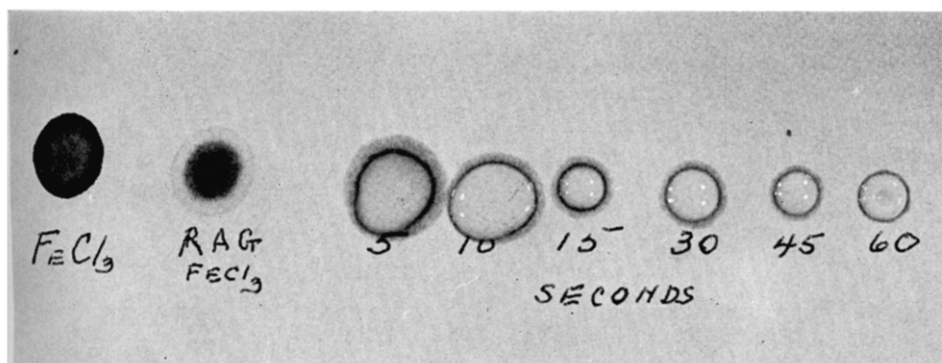


Fig. 1A

Reduction and elimination of the ferric chloride spot test with progressive emulsification. Reading from left to right are the spot tests produced by ferric chloride and the ferric chloride-ragweed combination. Successive spot tests at the indicated intervals show decreasing reactions with increased emulsification, until the reaction is completely obliterated in 60 seconds. When photographed some syneresis had already taken place since the droplet at the extreme right has a faint color. With complete emulsification this is eliminated.

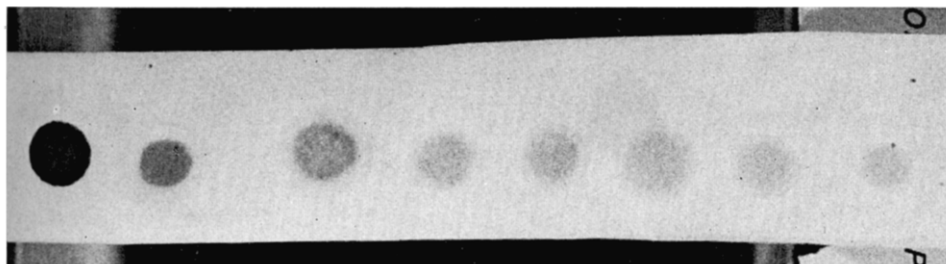


Fig. 1B

Spot test as seen on the reverse side of the filter paper. The emulsion is the same as in Fig. 1A.

addition of sufficient drops until the entire paper was treated. There is no need for any special filter paper; the paper strips used for hemoglobin determinations are suitable.

Chemical considerations

The chemical reaction involved in the test is the interaction of the ferric ion with the ferrocyanide ion, to produce the well-known Prussian blue reaction as shown by the formula:

$\text{Fe}^{+++} + \text{Fe}(\text{CN})_6^{----} \rightarrow \text{Fe}_4[\text{Fe}(\text{CN})_6]_3$ (Prussian blue). According to Feigl³ the limit of identification of the Fe^{+++} by this test is a dilution of 1:500,000.

The following experiments were conducted to evaluate the validity and accuracy of the spot test and to determine its usefulness in standardizing and monitoring W/O emulsions.

These data were evaluated statistically by the Pearson correlation coefficient and showed 100 per cent correlation between globule size of the emulsion and the spot test reaction. It is, therefore, possible to standardize emulsions on the basis of this test. A negative spot test invariably indicates globules ranging about 0.5 to 1.5 μ , regardless of the contents of the emulsion, whether pollen, LPS, molds, house dust, etc. A positive reaction was not observed unless the globules were over 4 μ in size. These observations were made with the ordinary light microscope with a calibrated micrometer in the eyepiece and need confirmation by more refined and accurate techniques. These emulsions were produced with the large Wig L Bug machine (Model 6,000)⁴ at intervals of 30 seconds, 60 seconds, and 3 minutes.

DISCUSSION

Water-in-oil emulsions have great potential for the administration of allergens, vaccines, and pharmacologic agents for prolonged action. In the case of allergens, lipopolysaccharides, and other potentially toxic agents, it is imperative that they be completely emulsified when made and when given, in order to avoid adverse reactions. For these purposes a chemical spot test, the first of its kind, was developed to evaluate and standardize W/O emulsions. The ferric chloride

Table I. *Relation of FeCl_3 spot test and emulsion globule size to survival of mice given a single lethal injection of emulsified lipopolysaccharide (LPS)*

Type of injection of LPS (2 mg.)*	Number of mice	Approximate range in diameter of globules (μ)	FeCl_3 † spot test	Survivors			
				12 hours		72 hours	
				No.	%	No.	%
Emulsified	8	0.5-1.5	0	8	100	7	87.5
	8	4-6	1+	8	100	6	75
	8	10-20	4+	7	87.5	0	0
Aqueous (unemulsified)	8	—	—	0	0	0	0

*The dose is about 4 times LD_{50} for the mouse (endotoxin of *E. coli* 026: B6).

†Reaction as determined by appearance and width of the blue border in the spot test (see text).

spot test described herein is accurate, simple, and rapid to perform. It eliminates the need for microscopy, conductance, or re-emulsification before injecting emulsified allergens. The test, however, is only qualitative and not quantitative. Perhaps with colorimetry it may ultimately be improved so as to provide more exact information about the amount of aqueous phase remaining unemulsified.

Ferric chloride was chosen in these studies, since it is nonantigenic, nontoxic, and readily detected by spot-testing in high dilution with potassium ferrocyanide. It does, however, lower the pH of the aqueous phase.

In the case of ragweed extract emulsions no apparent harm was done to the allergens, despite the fall in pH as shown by the clinical results,² by skin-testing, and by the induction of anaphylaxis in guinea pigs.⁵ It is apparent that in other situations other indicators and detectors may have to be employed, and many such combinations are possible.

We have also employed other ferric salts, such as ferric ammonium citrate and ferric ammonium tartrate, to avoid the fall in the pH of the extract produced by the FeCl_3 . Likewise, we have buffered the ferric chloride-treated extract with NaOH but find these modifications inadequate as substitutes for the FeCl_3 . Where neutral salts are used the spot test is not induced unless acid is added to the filter paper treated with the potassium ferrocyanide. Furthermore, the neutral salts do not produce as good emulsions as does FeCl_3 .

Ferric chloride has, therefore, added to the safety factor in the treatment of pollinosis, by enhancing and stabilizing emulsions of allergens. Samples of emulsified allergens prepared with ferric chloride have remained stable in the refrigerator for over two years.⁶ It is our contention that in vitro stability is at present the only criterion for anticipating in vivo stability.

We have also reversed the test by incorporating potassium ferrocyanide into the aqueous phase, with the ferric chloride applied to the test paper. Although the spot test was effective, the possible toxicity of potassium ferrocyanide deters its use clinically.

The ideal chemical to be employed in the detection of any unemulsified aqueous phase in W/O emulsions must satisfy the following criteria: it must be soluble in water but be completely insoluble in the oil; remain unaltered (or if altered exist in excess) for detection; be incapable of affecting adversely the agent to be emulsified; be incapable of reacting with the oil or interfering with the action of the emulsifier; and be capable of even distribution so that the result of the test drop would reflect the state of the entire emulsion. Ferric chloride as used in the spot test satisfies all these criteria.

Several questions about the reliability of the test may be raised. Does the test of a single drop reflect the status of the entire emulsion? Evidence presented here indicates that this is truly so. Can the test be relied upon in view of the failure of dilutions of ferric chloride greater than 1:1000 to elicit this test? This failure can be accounted for by the known chemical reaction of the ferric chloride in water in which tactoids of FeOH are formed, making the Fe^{+++} unavailable for reaction with the potassium ferrocyanide. This occurs at a pH of 2.6 and higher. (This, incidentally, explains the clouding of the ferric chloride solution upon standing.) This effect can readily be reversed by acidification with concentrated

HCl.⁷ At any rate, this phenomenon observed by the dilution of ferric chloride in no way reflects upon the validity of the spot test as described, in which no dilution occurs, nor does it invalidate the reported sensitivity of the test (1,500,000) if precautions regarding the pH are taken.

METHODS AND RESULTS

Correlation of the spot test with the degree of emulsification

Commercially available extracts of ragweed (giant and dwarf)* were diluted with buffered saline so that each milliliter contained 5,000 protein nitrogen units (PNU). To each milliliter of this solution was added 0.05 ml. of a 10 per cent solution of ferric chloride. This comprised the aqueous phase. When this solution was spot-tested on the filter paper, which had been pretreated with potassium ferrocyanide, an immediate reaction was obtained. The oil phase consisted of Drakeol 6 VR,[†] 88 ml.; Arlacel A[‡] (mannide monooleate), 10 ml.; and glyceryl monooleate,[§] 2 ml.¹

Two milliliters of the oil phase was first added to 5 ml. plastic vials and rotated so that the sides of the vial were coated with the oil. A similar amount of the aqueous phase was then added. The vial was capped, placed in a plastic holder, and shaken by the small Wig L Bug machine,^{||} with which it is possible to emulsify for intervals from 1 to 60 seconds.

In the experiment, emulsification was started and stopped at 5 to 15 second intervals; aliquots of the emulsion were removed by dropper and spot-tested until the test was negative.

In Fig. 1, one such test is depicted, and it is apparent that as the emulsion improves with prolonged shaking, the ferric chloride spot test reaction is lessened until it finally disappears when the emulsion is complete as verified by microscopy. Fig. 1*B* shows the spot tests on the reverse side of the filter paper.

Fig. 2 demonstrates syneresis of an emulsion by the filter paper as observed for one hour. It was originally negative, but with time and increased syneresis the spot test becomes larger and more intense.

Distribution of the ferric chloride in the emulsion

In the performance of the spot test, only one drop is employed. Does this reflect the entire state of the emulsion? Evidence that this is so may be obtained by repeatedly testing successive samples of a given emulsion, such as is done prior to injection of allergens. The samples were invariably negative if the initial test was negative.

Another more convincing demonstration of the validity of the test for the entire emulsion is shown in Fig. 3. Here, the entire contents of a syringe, containing 5 ml. of an emulsion of house dust extract, into which ferric chloride has been incorporated, was tested at one time. In the center of the filter paper im-

*Center Laboratories, Port Washington, N. Y.

[†]Pennsylvania Refining Co., Butler, Pa.

[‡]Hilltop Laboratories, Cincinnati, Ohio.

[§]Glyco Chemical Co., New York, N. Y.

^{||}Crescent Dental Manufacturing Co., Chicago, Ill. (Model 5A).

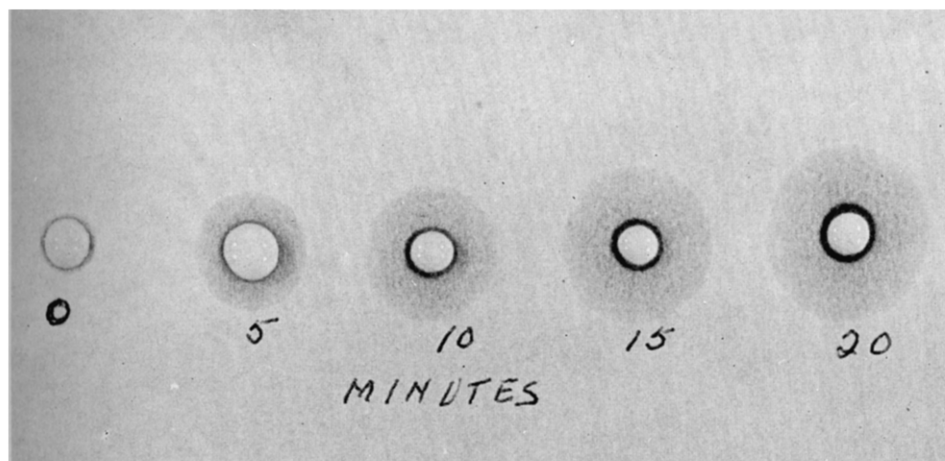


Fig. 2

Demulsification induced by syneresis of filter paper. Starting with a good emulsion and with a negative spot test, demulsification is induced by syneresis and is progressive with time. With continued syneresis the entire emulsion is ultimately disrupted. (Not shown in photo.)

pregnated with potassium ferrocyanide is a positive reaction, produced by a drop of FeCl_3 (10 per cent). Encircling this, in spirals, are the entire contents of the syringe, emptied like a tube of tooth paste. Fig. 3 is a photograph made immediately after emptying the syringe. No blue reaction is noted anywhere along the borders; the spot test for the entire emulsion is negative.

Accuracy of the test: It's usefulness in standardizing W/O emulsions

In evaluating the accuracy of the spot test, it was first compared with the crude tests commonly employed, such as gross appearance and drop-on-water test. Emulsions incorporating ferric chloride as an indicator were made and tested at 5 to 10 second intervals, after being shaken in the small Wig L Bug machine. When the emulsion on gross inspection looked good, and floated well on water, it still gave a positive spot test reaction. It was only after further emulsification that the spot test became negative. It is, therefore, apparent that the ferric chloride spot test is more sensitive in detecting the presence of the unemulsified aqueous phase.

Another demonstration of the sensitivity of the spot test is its use to detect demulsification. For this study eight samples were taken at random from sets of emulsions previously made with ragweed extracts containing FeCl_3 . The sets were part of a study in which various oils and emulsifiers were used singly and in combination. Grossly, they showed no signs of demulsification. When originally made they all were spot-test negative and were left standing at room temperature for various periods of time to observe for stability. When the eight selected emulsions were subjected simultaneously to the drop-on-water and spot test, it was observed that six emulsions yielded excellent stable floating globules but were positive by spot test, indicating demulsification. The remaining two emulsions were negative by spot test and the globules floated well on water.

**Fig. 3**

Spot test of entire emulsion. The content of a syringe of emulsion (5 ml.) is spot tested. There is no immediate reaction about the borders of the emulsion. In the center is a positive reaction obtained with a drop of ferric chloride solution (10 per cent).

It was apparent that with time demulsification had occurred in some emulsions, and that this was more readily detected by the spot test than by the drop-on-water test. Furthermore, it indicates how unreliable the gross appearance of an emulsion is.

Microscopy, on the other hand, can supply accurate information on the quality of an emulsion; the smaller the globules the better the emulsion.

In previous observations it had been noted that the best and most stable emulsions made were comprised of globules ranging about 0.5 to 1.5 μ in diameter. In view of this, experiments were performed in mice correlating the globule size and the ferric chloride spot test reaction, by observation of the lethal effect of an emulsion of lipopolysaccharide (LPS, *E. coli* 026:B6).^{*} This is shown in Table I.

Each of 32 mice was given 2 mg. of LPS (4 times the LD₅₀ dose) subcutaneously. Three groups of 8 received it in emulsions of various grades, while the fourth group, serving as a control, was given an aqueous suspension. There were no survivors in the control group at 12 hours, indicating extreme toxicity. On the other hand, there were survivors in the emulsion-treated groups, and these were directly related to globule size and to the spot test reaction. Thus, the best emulsion in which the globules were judged to be from 0.5 to 1.5 μ , and which

^{*}Difco Laboratories, Detroit, Mich.

gave a negative spot test reaction, yielded 100 per cent survivors in the first 12 hours and 87.5 per cent in 72 hours. In the second best emulsion, as determined by microscopic examination, the globules ranged from 4 to 6 μ , showed a slight blue border with the spot test, and resulted in a survival rate of 75 per cent in 72 hours. The third and poorest emulsion was comprised of globules ranging from 10 to 20 μ and gave a strong spot test reaction, characterized by a broad and intense blue ring about the droplet. With this emulsion there were 87.5 per cent survivors in 12 hours but none in 72 hours. It is noteworthy that with this emulsion, although inadequate, there was a high survival rate in the 12 hour period (87.5 per cent) compared with none for the same period in the control group. It would seem that even the poorest of emulsions is better than none in retarding absorption. This experiment also illustrates the use of a biologic test in evaluating a given emulsion.

A more fundamental question pertains to the possible impairment of ragweed antigenicity by ferric chloride. The induction of systemic reactions and positive skin tests in patients, and anaphylaxis in guinea pigs, suggests no impairment.⁶ King, Norman, and Connell,⁸ however, have demonstrated by gel diffusion an adverse effect on antigen E by the addition of acid, while Wodehouse¹⁰ reported the elimination of band "e." On the other hand, Rockwell⁹ used HCl to fractionate ragweed extract and showed, as we did, no impairment of its ability to induce anaphylaxis, elicit skin tests, and be effective therapeutically. Preliminary gel diffusion studies made in our laboratory with ferric chloride-treated extracts and the antisera produced by them in two rabbits suggest no major impairment of ragweed antigenicity, but additional studies in many more animals are needed to clarify the situation.

There is one limitation of the spot test which needs to be emphasized. It may not be useful in emulsions made with vegetable oils. Ferric chloride in this instance, although helpful in enhancing emulsification of systems normally difficult to emulsify (by formation of iron soap) remains in the oily outer phase, so that despite the excellence of the emulsion a strong positive reaction is obtained. Here, the ferric chloride in no way reflects the state of emulsification of the inner aqueous phase.

The technical standardization of emulsions of allergens must be a prime objective in the evaluation of the repository procedure. Without such standardization it is impossible to evaluate therapeutic results and the incidence of allergic reactions. No comparison can be made scientifically of the findings reported from different research groups without such a standard of reference. Nor can adequate double-blind studies be conducted without assurance that the emulsion as made and when administered is the same in quality for each patient. Such standardization we believe has been achieved with the application of the ferric chloride spot test. It correlates with microscopy 100 per cent; a negative spot test under the conditions described invariably indicates an excellent emulsion with emulsion globules ranging about 0.5 to 1.5 μ in diameter. In a recent collaborative study in four clinics, in which over 300 patients were treated for ragweed pollinosis with a centrally made emulsion, the test proved its reliability.² With the test as a guide, it was assured that all patients received qualitatively the same emulsion,

which simplified the analysis of the results achieved. As a safety check and time-saver, it should be a boon to the allergist employing the repository technique.

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