Colorimetric Analysis of Hexachlorophene in Topical Formulations

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Abstract □ The commonly used 4-aminoantipyrine dye formation procedure for hexachlorophene analysis in topical formulations was modified to overcome interference due to other components. Bar soaps and nonemulsion formulations are analyzed directly, employing a chloroform back-extraction stage of the dye prior to quantitation. Hexachlorophene in emulsions and liquid soaps is determined using a TLC separation prior to dye formation.

Keyphrases □ Hexachlorophene—colorimetric analysis in topical formulations, modified 4-aminoantipyrine dye formation method, elimination of interferences □ 4-Aminoantipyrine—dye formation for hexachlorophene colorimetric determination, modifications to overcome interferences □ Colorimetry—analysis, hexachlorophene in topical formulations, 4-aminoantipyrine dye formation, modified to eliminate interferences

Direct UV spectrophotometry (1, 2), absorbance measurements after sample cleanup (3, 4), NMR spectroscopy (5), GLC (6), and, more recently, high speed liquid chromatography (7) have been used to quantitate hexachlorophene (I) in topical formulations. However, the colorimetric determination of the dye formed when I reacts with 4-aminoantipyrine (II) (8–10) is the method most commonly used for analyzing such preparations (11–13).

The 4-aminoantipyrine procedure for analysis of I gives rise to difficulties due to instability of the dye formed (11, 14) and to interference from surfactants present in preparations (15). This report describes a modified II method for the analysis of I which overcomes many of these difficulties. The TLC procedures also described allow the application of the colorimetric method to emulsions.

EXPERIMENTAL[1]

Weak Ammonia Buffer (11)—Dissolve 67.5 g of ammonium chloride in 570 ml of concentrated ammonium hydroxide, dilute to 1 liter with distilled water, and mix. Dilute 2.0 ml of this solution to 1 liter with distilled water to afford the required weak ammonia buffer.

General Procedure (A)—*4-Aminoantipyrine Solution*—Prepare a 0.5% (w/v) solution of II in distilled water.

Potassium Ferricyanide Solution—Prepare a 2.0% (w/v) solution of potassium ferricyanide in distilled water.

Hexachlorophene Standards—Dissolve about 25 mg of reference standard quality I, accurately weighed, in 100.0 ml of dimethylformamide and mix. To individual 25-ml volumetric flasks, add, respectively, 2.0, 3.0, 4.0, and 5.0 ml of this solution and dilute to volume with dimethylformamide.

Sample Preparation—Transfer an accurately weighed aliquot of powdered bar soap, toothpaste, or dusting powder equivalent to about 0.55 mg of I to a centrifuge tube. Add 15.0 ml of dimethylformamide and mix thoroughly for 45 min using a mechanical shaker. Centrifuge or filter the solution to remove insoluble material.

Color Development and Hexachlorophene Determination—Transfer 1.0 ml of solution containing standard or sample to a 25-ml volumetric flask, add 20 ml of weak ammonia buffer and 1.0 ml of II reagent, and mix. Add 1.0 ml of potassium ferricyanide reagent, immediately dilute to volume with weak ammonia buffer, and again mix. Immediately transfer 15.0 ml of this solution to a 60-ml separator containing about 6 g of sodium chloride. Add 7.0 ml of chloroform and shake for 1–2 min. Allow layers to separate and collect most of the chloroform layer.

Filter the chloroform solution through a filter[2] into a 1.0-cm spectrophotometer cell. Measure the absorbance of the sample solution at 483 nm against a blank prepared in an identical manner but substitute 1.0 ml of dimethylformamide for solutions containing I.

Prepare a calibration curve plotting absorbances of standard solutions against micrograms per milliliter of I in the final chloroform solution.

Calculations—Calculate the percent of I using:

$$\% \text{ w/v I} = \frac{Cs \times 175}{Ws \times 10} \qquad \text{(Eq. 1)}$$

where Cs = micrograms per milliliter of I from the graph, and Ws = weight of sample taken in milligrams.

Chromatographic Method (B)—*Buffered 4-Aminoantipyrine Solution*—Prepare a 0.5% (w/v) solution of II in weak ammonia buffer.

Buffered Potassium Ferricyanide Solution—Prepare a 2.0% (w/v) solution of potassium ferricyanide in weak ammonia buffer.

Hexachlorophene Standards—Dissolve about 25 mg of reference standard quality I, accurately weighed, in ethanol and dilute to 25.0 ml.

Sample Preparation—Transfer to a centrifuge tube an accurately weighed aliquot of emulsion, solution, or other sample equivalent to about 10 mg of I. Add 10.0 ml of ethanol and mix thoroughly for 45 min, employing a mechanical shaker. If necessary, centrifuge or filter the resultant solution to remove insoluble material.

TLC and Isolation Procedure—Use self-prepared or precoated fluorescent layers of silica gel, 250 μm thick.

On a line 1 cm from one edge of the silica gel layer, apply duplicate spots using 10.0, 15.0, and 20.0 μl of standard solutions of I, alternating such spots with three others prepared with 15.0 μl of sample solution. Develop the plate for 15 cm in a paper-lined chamber, using ethyl acetate–methanol (9:1) (4) as the solvent.

Air dry the plate and examine it under shortwave UV light. Delineate I spots and collect silica gel from individual spot areas using a suitable apparatus (16). Isolate I by washing the silica gel with 6 ml of ethanol, collecting the solvent in a suitable tube or flask. Evaporate the ethanol to dryness.

Color Development and Hexachlorophene Determination—Carefully dissolve the residue obtained from silica gel extraction in 0.5 ml of ethanol and add 13.0 ml of weak ammonia buffer and 1.0 ml of buffered II solution. Mix, add 1.0 ml of buffered potassium ferricyanide solution, and again mix. To the resulting solution, immediately add about 6 g of sodium chloride and 7.0 ml of chloroform and shake vigorously for 1–2 min. Allow the layers to separate.

Using a 10-ml syringe and suitable needle, draw off the chloroform layer without delay. Pass this solution through a filter[2] into a 1.0-cm cell; measure the absorbance at 483 nm against a blank pre-

[1] Chemicals of analytical reagent grade or the equivalent are used. Ethanol means 95% ethanol.

[2] Filter holder SX 00013000 and filters PHWP01300, Millipore Corp., Bedford, Mass.

Table I—Analysis of Hexachlorophene in Topical Formulations by the General Colorimetric Procedure

Sample	Labeled Amount, w/w %	Found Label Claim, w/w %	Coefficient of Variation	Number of Determinations[a]
Simulated soap	2.0	97.56	1.60	8
A soap	2.0	101.05	1.93	5
B soap	2.16	101.4	0.92	3
C soap	Unlabeled	2.16[b]	1.70	3
D douche	2.0	102.05	0.51	6
E powder	0.5	100.63	2.91	6
F tooth-paste	Unlabeled	0.045[b]	3.33	4
G liquid soap	0.4	90.15	2.14	3

[a] Each determination constituted a separate weighing followed by color development in triplicate. [b] Percent on a weight basis.

Table II—Analysis of Topical Emulsions by the Specific Colorimetric Procedure

Sample	Labeled Amount, %	Percent Recovery, General Colorimetric Method	Percent Recovery, UV Absorbance	TLC/Colorimetric		
				Mean Percent Recovery	Coefficient of Variation	Number of Plates (Duplicate Spots)
G	0.4	90.15	104.4	103.17	0.43	2
H	0.7	89.47	108.7	105.2	2.74	3
I	0.5	94.80	163.1	94.5	2.08	3
J	3.0	96.89	105.5	105.7	2.08	3

pared as described but using a similar portion of the same silica gel layer *that contains no I*. Prepare a calibration curve plotting absorbances of standard solutions against micrograms per milliliter of I in the final chloroform solution and determine the content of I in the samples.

Calculations—The percent of I is calculated using Eq. 2:

$$\% \text{ w/w I} = \frac{Cs \times 525}{Ws} \qquad \text{(Eq. 2)}$$

where Cs = micrograms per milliliter of I from the graph, and Ws = weight of sample taken in milligrams.

DISCUSSION

Although phenols are commonly analyzed by the II color procedure, the dyes formed during color development are unstable in the alkaline solutions in which they must be generated (11, 14). This problem has been overcome for many phenols by extraction of the dye into nonpolar solvents such as chloroform (11, 14). This procedure was reported to be inapplicable to analysis of I because of the insolubility of the I–II dye in chloroform (11, 14). The present investigation indicated that this dye could be taken up in chloroform by "salting out" from the aqueous solution with common salt.

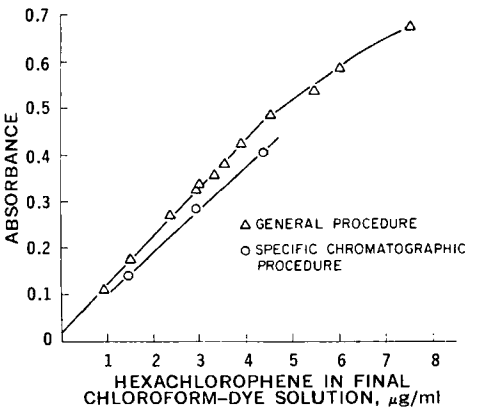

Figure 1—*Hexachlorophene calibration curves.*

The partition coefficient of the I–II dye between brine and chloroform appears to favor the latter. Solubility of the dye in chloroform was found to be in excess of 4.5 μg/ml. However, at concentrations over this level, volumes and shaking times became critical so that it was difficult to obtain reproducible results. Thus, in method development, sample sizes and solvent volumes were adjusted to keep I levels below 4.5 μg/ml.

Various aqueous phase–chloroform ratios were examined for extracting I–II dye into the chloroform layer. While the dye appeared only in the organic layer with ratios as low as 3:1, 15 ml of aqueous phase to 7 ml of chloroform was most convenient because of subsequent manipulation of the organic layer.

Using the color development and partitioning procedure, which subsequently became the proposed method, various concentrations of I were examined to determine whether dye solutions obeyed Beer's law at the absorbance maximum, 483 nm. Figure 1 demonstrates the direct relationship between concentration and absorbance (and also shows solubility characteristics of the I–II dye in chloroform).

Based on these considerations, the *General Procedure* for I analysis was developed for the examination of samples. The sample size proposed provides a I concentration in the final I–II dye solution of about 3.1 μg/ml and an absorbance near 0.33. This ensures that specimens containing overages of up to 150% will be in the linear range of the graph. Similarly samples containing as little as 50% of the label claim for I also will be in the accurate and precise range of the graph. All this, of course, assumes no interference from other components in formulations.

Since there are reports that analysis of I by II dye formation is adversely affected by soap and other surfactants (15), various organic solvents were examined to find one that would, ideally, isolate I and leave behind interfering substances from soap and other topical formulations. Chloroform did not isolate all of the I present in simulated samples while ethanol was nonselective, dissolving fatty acid salts and other materials so that lower than expected absorbances were obtained. Dimethylformamide, which has been used previously in I analysis (1), was found to be a good solvent for I in bar soap, dusting powder, and toothpaste but left some ambiguity when used with emulsion formulations.

Dimethylformamide was used in the isolation of I from a number of specimens of soap and other products (Table I). The coefficients of variation observed with some samples reflect difficulties in sampling (varying moisture levels, lack of homogeneity of specimens, *etc.*), since analysis of a solution of I in dimethylformamide

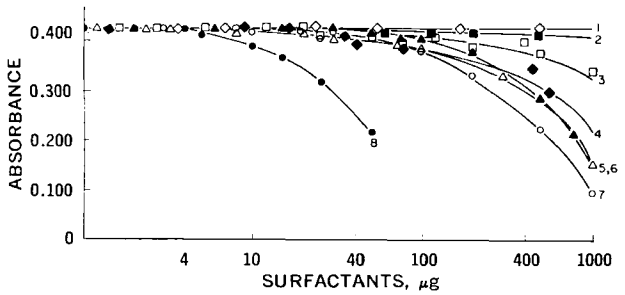

Figure 2—*Effects of surfactants on a constant concentration of 100 mg/25 ml hexachlorophene. Key: 1, ◇, sodium lauryl sulfate; 2, ■, sorbitan monooleate; 3, □, polyoxyethylene (23) lauryl ether; 4, ◆, polysorbate 80; 5, △, polyoxyl 30 stearate; 6, ▲, polyoxyl 8 stearate; 7, ○, polyoxyethylene (4) lauryl ether; and 8, ●, benzalkonium chloride.*

indicated recoveries of 97.9% with a coefficient of variation of 0.87.

Application of the proposed I method to emulsions left some doubt as to the accuracy of the procedure, since all values obtained were below labeled amounts (Table II). In addition, a liquid soap solution (Sample G) was low. To assess these results, Samples G–K inclusive were examined spectrophotometrically at 299 nm in acidified methanol. This study indicated higher I contents than were obtained by the II procedure (Table II).

These results, however, did not remove all ambiguity, since the absorbance curves for all samples showed the presence of background absorbance with maxima broader than those observed with equivalent concentrations of pure I. Thus, surfactants and other components could be interfering with both the II dye and direct UV procedures.

To determine the scope of the effects of surfactants on I analysis by II dye formation, solutions of I were treated with various levels of a number of commonly encountered surfactants, the II dye was developed, and absorbances were measured. Figure 2 demonstrates the limited tolerance of this colorimetric procedure to the presence of nonionic and cationic surfactants. In contrast, high levels of sodium lauryl sulfate did not interfere with color development.

It was apparent from this and other work (15) that it was necessary to isolate I from other emulsion components prior to color development. TLC on silica gel, with ethyl acetate–methanol (9:1) as solvent (4), was effective. In addition, this system separated I from other phenols such as dichlorophen, *o*-phenylphenol, 2,4-dichlorophenol, phenol, 2-benzyl-4-chlorophenol, 2,4,5-trichlorophenol, 4-chloro-3,5-dimethylphenol, and trichlorocarbanilide (Table III), so a high level of specificity could be obtained.

Direct scaling down of the general colorimetric procedure (A) for examining samples that had been chromatographed was not possible because of the limited buffering power of weak ammonia buffer. Where pH values of final solutions in the general procedure were always in the 9.45–9.67 range, direct scaling down afforded varying pH values with some as low as 8.35. Preparation of II and potassium ferricyanide reagents in buffer overcame this problem so that final values for the proposed specific chromatographic procedure (B) were always between 9.5 and 9.7.

TLC of standard solutions of I with subsequent isolation of I and color development indicated that 86% I was consistently recovered from chromatographic layers. Using this method, emulsion Samples H–J inclusive and the liquid soap solution G were analyzed (Table II).

Data derived from the *Chromatographic Method* were in good agreement with that obtained from UV absorbance measurements with the exception of Sample J (Table II). In this case, results obtained with Method B were very close to those obtained with the general colorimetric procedure. These data indicate that results obtained from general procedures, either colorimetric or UV, for quantitating I in emulsions and liquid soaps cannot be considered reliable. Thus, prior to utilizing a general method, the specific procedure must be applied to establish reliability. These problems do not arise with bar soap since both the general and specific colorimetric procedures afforded similar values.

Table III—Typical R_f Values of Antimicrobial Agents Examined

Compounds	R_f
Hexachlorophene	0.42
Trichlorocarbanilide	0.58
Phenol	0.62
2,4-Dichlorophenol	0.62
2,4,5-Trichlorophenol	0.62
o-Phenylphenol	0.63
4-Chloro-3,5-dimethylphenol	0.63
Dichlorophen	0.63
2-Benzyl-4-chlorophenol	0.64

The proposed modified colorimetric procedures for I analysis enables one to analyze occasional or replicate samples of bar soap and solutions containing I with minimum preparation time, while the specific procedure described enables the examination of emulsions with a high level of accuracy.

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