

Color Tests

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Color Tests

1 INTRODUCTION

Color tests are rapid toxicological qualitative methods used to establish the presence or absence of certain drugs based upon visible observation. The tests are primarily used to screen for the presence of drugs or metabolites in urine, but may also be used for other biological fluids (e.g., precipitated blood) and nonbiological samples (e.g., solids or liquids).

2 SCOPE

Analyses	<input checked="" type="checkbox"/> Screening <input type="checkbox"/> Confirmation <input type="checkbox"/> Quantitation	
Matrices	Urine	Blood
Analytes	Acetaminophen Salicylic acid Trihalomethyl Compounds (including chloral hydrate and metabolites) Ethchlorvynol ¹	Acetaminophen Salicylic acid
Personnel	This document applies to authorized personnel who perform the described tasks, singly or in combination.	

3 PRINCIPLE

Color tests involve the reaction of a specimen with a reagent or series of reagents to produce a change in color. The stronger the color change, the more drug or drug metabolite present in the sample.

- Acetaminophen and its metabolites are hydrolyzed in an acidic solution to p-aminophenol, which is coupled with o-cresol to give an indophenol blue color.
- Aspirin is hydrolyzed in an acidic solution to salicylic acid, which reacts with acidic ferric chloride solution to give a purple color.
- Trichlorinated compounds are polyhalogenated compounds. Compounds with at least two halogen atoms bound to the same carbon atom will react with pyridine to give a pink or deep yellow/orange color.
- Ethchlorvynol reacts with diphenylamine to form a purple color.

4 SPECIMEN CRITERIA

A minimum of 1.7 mL of urine is needed to perform all four color tests. A minimum of 2.0 mL of blood is required to perform the salicylate and acetaminophen color tests.

5 EQUIPMENT

5.1 Equipment

A. Hotplate – stirrer or Heating Block
B. Pipettes (0.5 - 5 mL, 200-1000 µL, 50-200 µL)

¹ As of the date of this procedure, ethchlorvynol is not available on the legal drug market.

C. Vortexer
D. Balance
E. Volumetric Flasks
F. Centrifuge
G. Evaporator with nitrogen
H. Digital Camera

5.2 Consumables

A. Pipette tips
B. Test tubes (13 x 100 mm)
C. Test tubes (16 x 100 mm)
D. Test tubes (12 x 75 mm)
E. 0.45 µm Millipore Filter Tubes

5.3 Chemicals/Reagents

5.3.1 Purchased

Stability and storage determined by manufacturer.

A. pyridine (certified ACS grade)
B. chloroform (HPLC grade)
C. methanol (HPLC Grade)
D. acetonitrile (HPLC Grade)
E. hydrochloric acid, concentrated
F. ammonium hydroxide, concentrated
G. o-cresol
H. mercuric chloride
I. ferric nitrate nonahydrate
J. sodium hydroxide
K. Diphenylamine (reagent grade)
L. Sulfuric acid (concentrated, 18M)
M. Deionized water

5.3.2 Prepared

A. 6 M Hydrochloric Acid (~ 50% v:v)
To a 25-mL graduated cylinder containing 10 mL deionized water, add 12 mL concentrated hydrochloric acid and mix well. Bring to 24 mL with deionized water. Store in glass at room temperature. Stable 6 months.
B. 2 M Ammonium Hydroxide
Add 10 mL concentrated ammonium hydroxide to 50 mL deionized water in a 100-mL graduated cylinder. Fill to the 75-mL mark with deionized water and mix well. Store in glass at room temperature. Stable 1 month.

C. 1% (by volume) o-Cresol
Place 1 mL o-cresol in a 100-mL volumetric flask and fill to the mark with deionized water. Mix well and allow to stand for at least 24 hours before use. Store refrigerated in brown glass. Stable 6 months.
D. Trinder's Reagent
Add 400 mg mercuric chloride, 400 mg ferric nitrate nonahydrate, and 0.1 mL concentrated hydrochloric acid to 5 mL deionized water in a 10-mL volumetric flask. Mix well to dissolve and bring to volume with deionized water. Store refrigerated in glass. Stable 3 months.
E. 5 M (20% w/v) Sodium Hydroxide
To a 100-mL Nalgene volumetric flask, add 60 mL water and 20 g sodium hydroxide. Mix well to dissolve and bring to volume with deionized water. Store in Nalgene containers at room temperature. Stable 1 year.
F. Diphenylamine Reagent
Diphenylamine Reagent (0.5% w:v in sulfuric acid): Dissolve 0.5 g diphenylamine in 100 mL concentrated sulfuric acid. Store in glass with a PTFE-lined cap at room temperature. Stable 1 year

5.4 Standards/Controls

5.4.1 Purchased

Stability and storage determined by manufacturer unless otherwise noted.

A. Negative Control Urine
Purchased or obtained in house ² . Store refrigerated in plastic.
B. Negative Control Blood
Purchased or obtained in house. Store refrigerated in glass or plastic.
C. Acetaminophen
D. Sodium Salicylate
E. Trichloroacetic Acid
F. Ethychlorvynol

5.4.2 Prepared

A. Acetaminophen Working Stock Solution (1 mg/mL)
Place 10 mg acetaminophen in 10-mL volumetric flask, dilute to mark with methanol. Store refrigerated in glass, stable at least 6 months.
B. Acetaminophen Positive Control Urine (100 µg/mL)
Add 1 mL of the Acetaminophen Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Urine. Store refrigerated in glass or plastic. Stable at least 6 months.
C. Acetaminophen Positive Control Blood (50 µg/mL)

² Note: Synthetic urine (Surine) is unsuitable for the salicylate color test.

Add 0.5 mL of the Acetaminophen Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Blood. Store refrigerated in glass or plastic. Stable at least 6 months.

D. Sodium Salicylate Working Stock Solution (1 mg/mL)

Place 10 mg sodium salicylate in 10-mL volumetric flask, dilute to mark with deionized water. Store refrigerated in glass. Stable at least 6 months.

E. Sodium Salicylate Positive Control Urine (100 µg/mL)

Add 1 mL of the Sodium Salicylate Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Urine. Store refrigerated in glass or plastic. Stable at least 6 months.

F. Sodium Salicylate Positive Control Blood (50 µg/mL)

Add 0.5 mL of the Sodium Salicylate Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Blood. Store refrigerated in glass or plastic. Stable at least 6 months.

G. Trichloroacetic Acid Working Stock Solution (1 mg/mL)

Place 10 mg trichloroacetic acid in 10-mL volumetric flask, dilute to mark with methanol. Store refrigerated in glass, stable at least 6 months.

H. Trichloroacetic Acid Positive Control Urine (50 µg/mL)

Add 0.5 mL of the Trichloroacetic Acid Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Urine. Store refrigerated in glass or plastic. Stable at least 6 months.

I. Ethylchlorvynol Working Stock Solution (100 µg/mL)

Place 100 µL ethylchlorvynol in a 12 x 75 mm test tube, add 900 µL of methanol. Store refrigerated in glass, stable at least 6 months.








J. Ethchlorvynol Positive Control Urine (30 µg/mL)

Add 1 mL of the Ethchlorvynol Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Urine. Store refrigerated in glass or plastic. Stable at least 6 months.






6 PROCEDURE

Results of the following tests should be recorded by photography.





6.1 Acetaminophen Color Test

	A. Preparation of blood samples:	Note	Reference/Lot
<input type="checkbox"/>	1. Add 1 mL of blood (sample or control) to a 16 x 100 test tube.	 2	
<input type="checkbox"/>	2. While vortexing, add 2 mL acetonitrile, dropwise.		
<input type="checkbox"/>	3. Transfer supernatant to a 0.45 µm Millipore filter tube.		
<input type="checkbox"/>	4. Centrifuge at approximately 4000 rpm for 15 minutes.		
<input type="checkbox"/>	5. Transfer filtrate to a new test tube, and evaporate to dryness under nitrogen at 35°C.		
<input type="checkbox"/>	6. Reconstitute residue in 0.5 mL deionized water. Vortex well.		
<input type="checkbox"/>	B. Place 0.5 mL of urine or reconstituted blood extract (sample or control) in a properly labeled test tube (16 x 100 mm). [Note: test also works at 1/5 volumes in a 10 x 75 mm test tube.]		
<input type="checkbox"/>	C. Add 0.5 mL of 6 M Hydrochloric Acid Solution to sample.		
<input type="checkbox"/>	D. Vortex samples for 10 - 15 seconds, and then place them in a boiling hot water bath or heating block at 98°C. Remove from water bath/block after 10 minutes.		
<input type="checkbox"/>	E. Add 3 mL of deionized water.		
<input type="checkbox"/>	F. Add 2 mL of 2 M Ammonium Hydroxide Solution.		
	G. Add 0.5 mL of O-cresol Solution (1%).		
<input type="checkbox"/>	H. Wait 10 - 25 minutes. (Wait a minimum of 25 minutes if acetaminophen and salicylate are expected in a blood extract.)		
<input type="checkbox"/>	I. View color, record. A blue color (indicative of acetaminophen) will appear with a positive result.		





6.2 Salicylate Color Test - (Trinder's color test)

	A. Preparation of blood samples:	Note	Reference/Lot
<input type="checkbox"/>	1. Add 1 mL of blood (sample or control) to a 13 x 100 test tube.	 2	
<input type="checkbox"/>	2. While vortexing, add 2 mL acetonitrile, dropwise.		
<input type="checkbox"/>	3. Transfer supernatant to a 0.45 mm Millipore filter tube.		
<input type="checkbox"/>	4. Centrifuge at approximately 4000 rpm for 15 minutes.		
<input type="checkbox"/>	5. Transfer filtrate to a new test tube, and evaporate to dryness under nitrogen at 35°C.		
<input type="checkbox"/>	6. Reconstitute residue in 0.2 mL deionized water. Vortex well.		
<input type="checkbox"/>	B. Place 0.2 mL of urine or reconstituted blood extract (sample or control) in a properly labeled test tube (12 x 75 mm).		
<input type="checkbox"/>	C. Add 0.2 mL of Trinder's Reagent.		
<input type="checkbox"/>	D. Vortex for 10 - 15 seconds.		
<input type="checkbox"/>	E. Wait 5 minutes.		
<input type="checkbox"/>	F. View color. A tan/brown (indicative of methylsalicylate) or tan/purple (indicative of sodium salicylate) will appear with a positive result.		

6.3 Trichlorinated Compound Color Test - (Fujiwara color test)

		Note	Reference/Lot
<input type="checkbox"/>	A. Place 0.5 mL of urine (sample or control) in a properly labeled test tube (12 x 75 mm).	 2	
<input type="checkbox"/>	B. Add 0.5 mL of 20% Sodium Hydroxide Solution.		
<input type="checkbox"/>	C. Add 0.5 mL of pyridine.		
<input type="checkbox"/>	D. Vortex samples for 10 - 15 seconds, and then place them in a boiling hot water bath or heating block at 98°C. Remove from water bath/block after 10 minutes.		
<input type="checkbox"/>	E. View color in the top (pyridine) layer. A pink (indicative of chloral hydrate or trichloroacetic acid) or deep yellow/orange (indicative of 2,2,2-trichloroethanol) will appear with a positive result.		

6.4 Ethchlorvynol Color Test

		Note	Reference/Lot
<input type="checkbox"/>	A. Place 0.5 mL of urine (sample or control) in a properly labeled test tube (12 x 75 mm).	 _2	
<input type="checkbox"/>	B. Add 0.25 mL of diphenylamine reagent.		
<input type="checkbox"/>	C. Vortex for 10 - 15 seconds.		
<input type="checkbox"/>	D. Wait 5 minutes.		
<input type="checkbox"/>	E. Add 1 mL of chloroform.		
<input type="checkbox"/>	F. Vortex samples for 10 - 15 seconds.		
<input type="checkbox"/>	G. Wait approximately 1 minute.		
<input type="checkbox"/>	H. View color. A purple color (indicative of ethchlorvynol) will appear in the lower (chloroform) layer with a positive result, or blue (indicative of negative specimen) will appear in the lower (chloroform) layer with a negative result.		

7 DATA ANALYSIS

7.1 Decision Criteria

Generally speaking, a specimen is considered to give a positive response for a particular drug class if the specimen compares favorably to the corresponding positive control. A negative response lacks the corresponding color change.

A. Acetaminophen Color Test:

1. Blue color indicates positive result for acetaminophen.

B. Salicylate Color Test:

1. Tan/brown color indicates a positive result for methylsalicylate.
2. Tan/purple color indicates a positive result for sodium salicylate.

C. Trichlorinated compounds Color Test:

1. Pink (or very deep red-purple for strong positive) color in upper (pyridine) layer within minutes indicates a positive result for chloral hydrate or trichloroacetic acid.
2. Deep yellow/orange color in top layer indicates a positive result for 2,2,2-trichloroethanol.
3. Note: May not be a good indicator for 2,2,2-trichloroethanol since positive result (deep yellow/orange color) may be hard to differentiate from the color of urine specimen, even with analysis of a negative control.

D. Ethchlorvynol Color Test:

1. Purple color in the lower layer within 2-3 minutes indicates a positive result for ethchlorvynol.

If a specimen is deemed positive for a particular drug class, a separate analysis utilizing a different principle must be performed in order to confirm the presumptive positive.

7.2 Control Criteria

Negative and positive controls shall be clearly observed to give different color changes.

8 REPORTING

Positive results of a color test are only preliminary. A more specific analytical method must be used in order to obtain a confirmed analytical result. Gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS) are preferred confirmatory methods.

9 CORRECTIVE MEASURES

Reference TOX-101 11.3.

10 PERFORMANCE CHARACTERISTICS

10.1 LOD

- A. Acetaminophen Color Test:
 - 1. 40 µg/mL for acetaminophen in urine
 - 2. 10 µg/mL for acetaminophen in blood
- B. Salicylate Color Test:
 - 1. 300 µg/mL for methylsalicylate in urine
 - 2. 45 µg/mL for sodium salicylate in urine
 - 3. 10 µg/mL for sodium salicylate in blood
- C. Trichlorinated Compounds Color Test:
 - 1. 10 µg/mL chloral hydrate
 - 2. 4 µg/mL trichloroacetic acid
- D. Ethchlorvynol Color Test:
 - 1. 25 µg/mL ethchlorvynol

11 LIMITATIONS

11.1 Sensitivity

- A. Acetaminophen, Salicylate, and Ethchlorvynol Color Tests:
 - 1. Positive results can be obtained with therapeutic doses.
- B. Trichlorinated Compounds Color Test:
 - 1. Positive results can be obtained with therapeutic doses and with overdoses in urine.

11.2 Specificity

- A. Acetaminophen Color Test:
 - 1. Positive results may be obtained with phenacetin (acetaminophen and p-aminophenol are metabolites of phenacetin, which is not available within the US).
 - 2. False negative results may occur in patients with ketonuria.
 - 3. When both acetaminophen and salicylate are present in a blood sample, false negative results may be obtained if the wait time is not lengthened to 25 minutes.
- B. Salicylate Color Test:
 - 1. False positive results may be obtained from the following compounds: aminosalicilylic acid, diflunisal, labetalol.
 - 2. When both acetaminophen and salicylate are present in a blood sample, false negative results may be obtained for low levels of salicylate (less than 50 PPM).

C. Trichlorinated Compounds Color Test:

1. Positive results indicate the presence of a polyhalogenated compounds. Polyhalogenated compounds that may also give positive results include: bromoform, carbon tetrachloride, chloramphenicol, chlorbutol, chlorobutanol, chloroform, DDT, ethyl bromide, ethylene chloride, iodoform, methyl bromide, methylene chloride, pentachloroethylene, perchloroethylene, tetrachloroethane, tribromoethanol, and trichloroethylene.

D. Ethchlorvynol Color Test:

1. False positive results may be obtained from phenothiazines.

12 SAFETY

Take standard precautions for the handling of chemicals and biological materials. Refer to the FBI Laboratory Safety Manual for proper handling and disposal of all chemicals.

13 REVISION HISTORY

Revision	Issued	Changes
03	02/11/2022	Section <u>1</u> – Moved text to Reporting . Section <u>2</u> – Updated scope statements. Section <u>4</u> – Removed unnecessary text. Section <u>5</u> - Updated order of equipment to blend QAM, OSAC and ABFT guidance. Removed duplicative text. Section <u>6</u> -Updated format. Updated rest of document for formatting improvements and clarification.