CONFIRMATION OF SALICYLATES IN BIOLOGICAL SPECIMENS

29.1 POLICY

This test method may be used to confirm the presence of salicylates in biological specimens. Any adjustments or deviations from the procedures below must be approved by the State Toxicologist, a Manager, or a Supervisor, and appropriately documented in the batch file.

29.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the confirmation of salicylates in biological specimens. This procedure will serve as the laboratory document describing sample preparation and criteria for acceptance and reporting of the specified compound.

29.3 PRINCIPLE

Concentration of salicylates is measured in whole blood, serum, plasma or urine by the Trinder's method. The specimen is treated with Trinder's reagent, an acidic solution of ferric nitrate and mercuric chloride. The free carboxylic acid of salicylate reacts with Fe^{3+} to form a violet complex with an absorbance at the 540 nm wavelength. Mercuric chloride causes precipitation of proteins, allowing for the violet colored supernatant to be tested by UV-Visible Spectrophotometry. Multiple-point calibration is used to generate a calibration curve. The concentration of salicylates identified in a sample is determined from its calibration curve.

29.4 SPECIMENS

29.4.1 The specimen volume is 0.5 mL.

29.4.2 Specimens include whole blood, serum, plasma or urine.

29.4.3 Dilutions of specimens may be analyzed at the Forensic Scientist's discretion; however, this should be done in addition to testing the standard specimen volume, unless sample quantity dictates otherwise.

29.4.4 Analysis of larger specimen volumes must be approved and documented.

29.5 REAGENTS, MATERIALS AND EQUIPMENT

29.5.1 REAGENTS

29.5.1.1 Certified blank blood

29.5.1.2 Deionized water (DI H_2O)

29.5.1.3 Ferric nitrate ($Fe(NO_3)_3 . 9H_2O$)

29.5.1.4 Hydrochloric acid (HCl, concentrated 12N)

29.5.1.5 1M Hydrochloric acid

Add 400 mL DI H_2O to a glass flask. Carefully add 42 mL concentrated HCl (12N). Dilute with DI H_2O to a final volume of 500 mL. Store at room temperature in a glass container for up to one

year. Volumes may be adjusted provided that the proportions remain constant.

29.5.1.6 Mercuric chloride ($HgCl_2$)

29.5.1.7 Methanol (MeOH)

29.5.1.8 Trinder's reagent

Dissolve 40 g $HgCl_2$ in 850 mL DI H_2O. Add 120 mL of 1M HCl. Add 40 g $Fe(NO_3)_3 . 9H_2O$ and apply magnetic stirring to dissolve. Dilute to a total volume of 1 L with DI H_2O. Store the solution at room temperature in a plastic bottle for up to one year. Volumes may be adjusted provided that the proportions remain constant.

29.5.2 MATERIALS

29.5.2.1 Cuvettes for UV-Vis spectrophotometer

29.5.2.2 Disposable 16 x 125 mm tubes with closures

29.5.2.3 Disposable pipette tips

29.5.2.4 Disposable transfer pipettes

29.5.2.5 Glass and plastic storage bottles

29.5.2.6 Laboratory glassware (graduated cylinders, flasks, beakers)

29.5.2.7 Magnetic stir bar

29.5.2.8 Magnetic stir plate

29.5.2.9 Volumetric glassware (flasks, pipettes)

29.5.3 EQUIPMENT

29.5.3.1 Calibrated, adjustable air-displacement pipettes

29.5.3.2 Centrifuge

29.5.3.3 Hewlett-Packard 8453 UV-Vis Spectrophotometer (UV-Vis)

29.5.3.4 Vortex mixer

29.6 STANDARDS, CALIBRATORS AND CONTROLS

29.6.1 STANDARD

29.6.1.1 Working standard (5 mg/mL)

a. Using an analytical balance, weigh out 50 mg salicyclic acid and add to a 10 mL class A volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the working standard is 5 mg/mL. The working standard is stored in an amber bottle in the freezer and expires one year from the date of preparation. Volumes may be adjusted, provided that proportions remain constant.

29.6.2 CALIBRATORS

29.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 29.7 SAMPLE PREPARATION. If necessary, calibrators may be prepared in alternate matrices provided that the matrix has been previously determined to not contain any of the compounds tested for by this procedure.

29.6.3 MATRIX BLANK AND CONTROLS

29.6.3.1 Matrix Blank

a. Analysis of certified blank blood is used as a matrix blank for the spectrophotometer to subtract out any contribution from the blood at the 540 nm wavelength.

29.6.3.2 Negative Control

a. At least one negative control is tested with every batch. The negative control is prepared using certified blank blood.
b. When testing different sample types, wherever possible, include a negative control prepared from that matrix. (For example, when analyzing whole blood and urine samples the batch shall include at least one negative whole blood control and at least one negative urine control.)

29.6.3.3 Positive Control (300 mg/L)

a. One positive whole blood control is tested with every batch. The positive control is prepared using certified blank blood to which the designated volume of control working standard has been added.
b. Control stock standard is obtained from an approved reference material supplier.
c. The control stock standard must be either a different lot number or from a different supplier to those used in producing the working standard. If the same lot or supplier must be used, the working control standard should be prepared by someone other than the person that prepared the working standard.
d. The control working standard (5 mg/mL) is prepared as described in 29.6.1.1.
e. The preparation of the positive whole blood control is detailed in 29.7 SAMPLE PREPARATION. Alternatively, quality control personnel may provide an in-house positive control.
f. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

29.7 SAMPLE PREPARATION

29.7.1 Label a clean 16 x 125 mm tube for each of the three calibrators, negative control, positive control, matrix blank and case samples.

29.7.2 Using the working standard, spike the calibrators according to the following table, adding the appropriate amount of working standard and certified blank blood as necessary. Vortex mix.

Calibrator Description	Volume Added (µL) Working Standard	Volume Added (µL) Blank Blood
Calibrator 1 (100 mg/L)	10	490
Calibrator 2 (500 mg/L)	50	450
Calibrator 3 (1000 mg/L)	100	400

29.7.3 Pipette 0.5 mL certified blank blood into the negative control tube and blank matrix tube.

29.7.4 Pipette 470 µL certified blank blood into the positive control tube.

29.7.5 Add 30 µL working control standard to the positive control tube.

29.7.6 Sample 0.5 mL of each case sample into its respective tube.

29.7.7 Add 4 mL Trinder's reagent to each tube, cap and vortex mix.

29.7.8 Centrifuge the tubes for 5 minutes at 2000 rpm.

QUALITATIVE COLOR TEST

A qualitative screen for the presence of salicylates in the specimen(s) may be performed. The matrix blank, calibrator 1 (100 mg/L), and the case specimen(s) are prepared as described in 29.7.

A comparison of the color change of the specimen(s) relative to the negative and calibrator 1 may be used to determine whether full sample preparation/analysis for confirmation and quantitation is necessary (see 29.12.5).

29.8 INSTRUMENTAL PARAMETERS

The instrumental parameters and instructions for operation of the UV-Vis can be found in Appendix A.

29.9 DATA ANALYSIS

29.9.1 Analysis of the batch data is conducted using the instrumental data analysis software associated with the UV-Vis.

29.9.2 Quantitative calculations are generated by multi-point, linear regression with equal weighting. The calibration curves are updated using the calibrator results for the batch; no historical calibration curves are permitted.

29.9.3 Summary reports are printed for the calibration, negative and positive controls and case samples. These reports are submitted for review.

29.9.4 Technical review of the batch is conducted according to the criteria listed below.

29.10 CRITERIA FOR BATCH ACCEPTANCE

If the analysis of the batch meets the criteria listed below, the results for the specimens are accepted.

29.10.1 Calibrators

29.10.1.1 Quantitative results for each calibrator should be within ± 20% of the target value. These are inclusive ranges. Result comparison is based on the whole number, truncated value in units of mg/L.

29.10.1.2 The calibration curve shall have a correlation coefficient of ≥ 0.99.

29.10.2 Controls

29.10.2.1 Negative Control

a. The negative control shall not identify salicylates at an absorbance of greater than 20% of that in calibrator 1.

29.10.2.2 Positive Control

a. Quantitative results for the positive control shall be within ± 20% of the target value. This is an inclusive range. Result comparison is based on the whole number, truncated value in units of mg/L.

29.11 CRITERIA FOR CASE SAMPLE ACCEPTANCE

If the criteria for batch acceptance have been satisfied, the results of individual case samples are deemed suitable for reporting if the following criteria are met.

29.11.1 The quantitative result must lie within the dynamic range of the method.

29.11.2 When dilutions of case samples are tested, the quantitative result(s) before multiplication shall be within the dynamic range of the test method.

29.12 REPORTING

29.12.1 Results are reported in units of milligrams per liter (mg/L).

29.12.2 Results are truncated to no more than two significant figures for reporting.

a. Example 1: concentration of salicylates is measured as 958.23600 mg/L.

b. The result is truncated to 950 mg/L (two significant figures) and reported.

c. Example 2: concentration of salicylates is measured at 501.03002 mg/L.

d. The result is truncated to 501 mg/L, but reported as 500 mg/L (one significant figure).

29.12.3 When multiple dilutions are analyzed, the smallest dilution within the dynamic range is reported.

29.12.4 If salicylates are initially identified using this method, they must be confirmed using this or another test method on a separate sampling before reporting.

29.12.5 Results of the qualitative color test may be reported as either negative or presumptive positive, as appropriate, using this colorimetric assay. If reported as presumptive positive, a separate sampling of the specimen will be tested using full sample preparation/analysis for confirmation and quantitation.

29.13 METHOD PERFORMANCE

29.13.1 Lower limit of quantification: 100 mg/L

29.13.2 Dynamic range: 100 mg/L – 1000 mg/L

29.13.3 Upper limit of quantification: 1000 mg/L

29.14 REFERENCES

29.14.1 Porter, W.H., Moyer, T.P.: Clinical Toxicology (Salicylate). *In*: Tietz Fundamentals of Clinical Chemistry, 4th ed., C.A. Burtis, E.R. Ashwood, Eds., W.B. Saunders, Philadelphia, 1996, pp. 440-442.

29.14.2 A Rapid and Simple Color Test for Detection of Salicylate in Whole Hemolyzed Blood. Asselin, W.M.; Caughlin, J.D. *J. Anal. Toxicol.* **1990**, 14, 254-255.

29.14.3 Widdop, B.: Hospital Toxicology and Drug Abuse Screening (Salicylic Acid). *In*: Clarke's Isolation and Identification of Drugs, 2nd ed., A.C. Moffat, J.V. Jackson, M.S. Moss, B. Widdop, Eds., The Pharmaceutical Press, London, 1986, p. 26.

29.14.4 Baselt, R.C.; Disposition of Toxic Drugs and Chemicals in Man, 6th ed., Biomedical Publications, Foster City, CA, 17-20.

APPENDIX A - Operation of the UV-Visible Spectrophotometer

1. Select the HP 8453 UV-Visible "ONILINE" icon and double click to start the software.
2. From the pull down menu, select Standard Mode.
3. From the Task tab on the menu, select Quantification.
4. Select the sampling method – Manual.
5. Verify that the salicylate method is loaded. It should be listed as "SALICYL.M" in the green method window in the top center of the screen.
6. If necessary, load the salicylate method under File>Load Method.
7. Turn on the tungsten lamp by clicking on it in the lower left corner. Allow the lamp to warm up for at least 30-45 minutes prior to analyzing the samples.
8. Select Method>Options & Info. Select Autosave Spectra to File. Name the file using the convention YYMMDDxx, where xx is the initials of the analyst.
9. Change the operator name under "Configuration".
10. Select a clean, un-scratched cuvette. Using a disposable transfer pipette, transfer some matrix blank supernatant into the cuvette. Dry off the outside of the cuvette with a Kim-Wipe, as necessary.
11. Place the blank sample in the cuvette chamber, and latch it in place.
12. Press the "Blank" button in the Sampling window.
13. The instrument will acquire a blank spectrum and display the Fourier transform spectrum of the blank. At any time during analysis, this spectrum can be displayed by selecting View>Last Blank Spectrum.
14. Remove the cuvette and either dispose of the blank solution in a beaker, or return it to the original test tube. Rinse the cuvette several times with fresh Trinder's reagent, and dump the waste into a beaker.
15. Transfer the supernatant of the first calibrator into the rinsed cuvette, and lock it in place.
16. Press the "Standard" button in the Sampling window. The HP 8453 will acquire the first standard spectrum and display a window to request the standard information. Input "Cal 1 (100 mg/L)"
17. The program will display the spectrum in the "Processed Standard Spectra" window, along with a "Calibration Table" window showing the standard curve.
18. Rinse the cuvette as in step 14.
19. Acquire the remaining two calibrators as in steps 14-16, using "Cal 2 (500 mg/L)" and "Cal 3 (1000 mg/L)" for the standard information.
20. Ensure that the resulting calibration coefficient is ≥ 0.99. If it is not, an attempt to reacquire some or all of the calibrators can be attempted, or the samples may need to be re-extracted.
21. After rinsing as in step 14, place the supernatant of the positive control into the rinsed cuvette, and press the "Sample" button in the Sampling window. The program will display the spectrum and a "Sample Table" window. Enter the sample information as "Pos Control (300 mg/L)". As you acquire successive sample spectra, they will be displayed in an overlay plot, and the table will automatically increase as required.
22. Collect the spectra for the negative control as in step 21, entering sample information as "Neg Control."
23. Collect the spectra(s) for any case samples as in step 21, entering the ST# for the sample information.
24. Print a summary report of the calibration on a color printer by selecting File>Print>Method.
25. Print a summary report of the negative control, positive control and case samples by selecting File>Print>Results.

26. Turn off the lamp under Instrument>Lamp(s)
27. Exit the program and turn off the computer.

LIST OF CHANGES

Revision Date	Description	Page Number
07/01/14	Method approved by Washington State Toxicologist. See DRA dated 06/24/14. Method released for use in evidentiary testing 07/01/14.	All