BENZIDINE in urine (SCREENING TEST) 8304

$C_{12}H_{12}N_2$ MW: 184.24 CAS: 92-87-5 RTECS: DC9625000

METHOD: 8304, Issue 2 **EVALUATION: PARTIAL** **Issue 1: 15 February 1984**
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BIOLOGICAL INDICATOR OF: exposures to benzidine-based azo dyes.

SYNONYMS: [1,1'-biphenyl]-4,4'-diamine

BIOLOGICAL SAMPLING		MEASUREMENT	
SPECIMEN:	2 urine samples, 150 mL each, before and after 6 h of exposure	**TECHNIQUE:**	VISIBLE ABSORPTION/THIN LAYER CHROMATOGRAPHY (TLC)
PRESERVATIVE:	none	**ANALYTE:**	2,4,5-trinitrobenzene sulfonic acid derivative of benzidine
SHIPMENT:	with dry ice in insulated container	**WAVELENGTH:**	400 nm
STABILITY:	stable for 2 months @ - 20 °C	**TLC IDENTIFICATION:**	UV and visible R_f = 0.41
CONTROLS:	collect urine from non-exposed workers	**ESTIMATED LOD:**	0.1 µg/100 mL urine (visible absorption); 0.3 µg/100 mL urine (TLC)
		CALIBRATION:	aqueous solutions of benzidine
		QUALITY CONTROL:	frozen pooled urine
		RANGE:	0.1 to 20 µg/100 mL urine
		RECOVERY:	70% @ 0.5 µg/100 mL urine
		PRECISION ($\bar{S}_r$):	0.12 @ 0.5 µg/100 mL urine
		ACCURACY:	± 53%

APPLICABILITY: This method is specific for aromatic amines and can be used to screen workers exposed to benzidine or benzidine-based azo dyes.

INTERFERENCES: In addition to false positives from other free aromatic amines, some drugs (e.g., antihistamines) contain free aromatic amines; however, these compounds do not produce R_f values corresponding to benzidine by TLC.

OTHER METHODS: This method replaces P&CAM 315 [1] with minor revisions. Method 8306 is a specific method for benzidine in urine by electron capture gas chromatography.

REAGENTS:

1. Methyl alcohol.
2. Benzidine, 99% (CAUTION: CARCINOGEN). *
3. Benzidine stock solution, 500 µg/mL. Weighed 50 mg benzidine. Dissolve in methyl alcohol to make 100 mL solution. Stable one month at -8°C.
4. Calibration stock solution, 10 µg/mL. * Dilute 1.00 mL benzidine stock solution to 50 mL with methyl alcohol. Prepare fresh daily.
5. Chloroform.
6. Hydrochloric acid.
   a. 1.0 N. Dilute 83 mL conc. HCl to 100 mL with distilled water.
   b. 0.1 N. Dilute 10 mL 1 N HCl to 100 mL with distilled water.
7. Sodium hydroxide, 1 N. Dissolve 40 g NaOH in water to make 1 L solution.
8. Sodium Chloride, crystals.
9. Sodium acetate buffer, 2 M, pH 5.5 Titrate 2 M sodium acetate with 6 N HCl. Refrigerate.
10. 2,4,6-Trinitrobenzene sulfonic acid (TNBS), 0.1 g/mL. Dissolve 2.5 g TNBS in 25 mL distilled water. Stable seven days when stored in the dark.
11. Acetone.
12. Formic acid.
13. Nitrogen, compressed.
14. Chloroform:formic acid, 90:10 (v/v). Prepare fresh daily.

* See Special Precautions.

EQUIPMENT:

1. Polyethylene bottles, 250-mL.
2. Spectrophotometer for measuring absorbance at 400 nm and 1-mL semi-microcuvettes.
3. Centrifuge, 400 rpm.
4. Rotator for mixing 25 × 200 mm test tubes.
5. pH meter.
6. TLC plates precoated with silica gel activated at 110 °C for 30 min., without flourescence indicator (0.5 mm thichness).
7. Chromatographic TLC tank.
8. UV source for reading TLC plates.
9. Glass bottles, 180-mL, with PTFE-lined caps.
10. Pipettes, glass, 2-, 5- and 100-mL, with pipet bulb.
11. Separatory funnels, 125-mL.
12. Volumetric flasks, 25- and 100-mL; 10-mL amber.
13. Culture tubes, glass with PTFE-lined caps (16 × 125 mm and 25 × 200 mm).
14. Micropipettes, 0.01-, 0.1- and 0.7-mL.
15. Pasteur pipettes.
16. Plastic gloves.
17. Dessicator.
18. pH paper (pH 2).

SPECIAL PRECAUTIONS: Benzidine is a known human carcinogen. Appropriate precautions should be utilized to minimize exposures.

All wastes, including acetone rinses of dirty glassware should be collected and disposed of by approved methods.

SAMPLING:

1. Take pre- and post-shift urine samples (ca. 150 mL) in 250-mL polyethylene bottles.
2. Ship the samples with dry ice in an insulated container.

SAMPLE PREPARATION:

3. Defrost sample if frozen. Adjust the urine pH to between 5 and 6 with 1 N HCl or 1 N NaOH.
4. Pipet 100 mL urine into a 180-mL glass bottle. Start a blank control urine sample (100 mL) and two control urine samples (100 mL) spiked with benzidine (0.3 to 1.0 µg) at this point.
5. Add 0.2 g NaCl to the pH-adjusted urine.
6. Extract the urine twice more with 10 mL chloroform for 2 min. If an emulsion forms centrifuge to seperate the two phases. Collect and save the chloroform fraction.

7. Extract the urine twice more with 10-mL portions of chloroform. Combine the three chloroform fractions.
8. Re-extract the combined chloroform mixture with 2 mL 0.1 N HCl for 30 min on a rotator.
9. Transfer the aqueous phase (ca. 2 mL) into a culture tube (16 × 125 mm) using a Pasteur pipet.
10. Add 2 mL pH 5.5 sodium acetate buffer and 0.7 mL of TNBS reagent, mix well, and let stand for 15 min at room temperature. Start reagent blank (2 mL 0.1 N HCl, 2 mL pH 5.5 sodium acetate buffer, and 0.7 mL TNBS reagent).

CALIBRATION AND QUALITY CONTROL:

11. Prepare a series of working standards in the range 0 to 20 µg benzidine/100 mL urine by adding aliquots of calibration stock solution to 100-mL portions of control urine (urine pool previously shown to have <0.1 µg benzidine/100 mL urine).
12. Prepare and analyze the working standards (steps 3 through 10 and 14 through 16).
13. Prepare a calibration graph, absorbance at 400 nm vs. concentration of analyte (µg/110 mL urine).

MEASUREMENT:

14. Add 2 mL $CHCl_3$ to the extract in step 10 and shake for 1 min.
15. Measure the absorbance vs. the reagent blank of the organic phase at 400 nm. Retain the organic phase for the benzidine-TLC confirmation (steps 16 through 19) if the sample contains more than 0.3 µg/100 mL.
16. Concentrate the organic phase containing the TNBS-amine derivative by evaporating with nitrigen to ca. 0.2 mL.
17. Spot 10 µL of the concentrated organic phase on an activated silica gel TLC plate.
18. Develop the plate in 90:10 chloroform:formic acid.
19. Compare the R_f of the unknown amine derivative with that of a benzidine-spiked derivative. Benzidine produces a spot with R_f = 0.41 which is yellow in visible light and dark under UV (254 nm) light.

CALCULATIONS:

20. Obtain the concentration of the analyte in the urine sample by comparing its absorbance with the calibration graph.
    NOTE: Corrections for extraction efficiency are not needed since standards are prepared in urine and both standards and samples are treated the same way.

GUIDES TO INTERPRETATION:

In this laboratory, normal ranges of urine specimens from NIOSH employees not exposed to benzidine or aromatic amines where:

Number of Urine Specimens	Aromatic Amine Conc. (µg/100 mL)
10	<0.1
2	0.1 to 0.2
1	0.2
1	0.3

Benzidine was not detected by TLC in any of the 14 urine specimens (LOD = 0.3 µg/100 mL).

EVALUATION METHOD:

Ten spiked urine specimens containing 0.5 µg benzidine/100 mL urine each were analyzed. Precision, S_r, for the 10 specimens was 0.12.

REFERENCES:

[1] NIOSH Manual of Analytical Methods, 2nd. ed., V. 5, P&CAM 315, U.S. Department of Health Education, and Welfare, Publ. (NIOSH) 79-141 (1979).

METHOD WRITTEN BY:

William P. Tolos, NIOSH/DBBS.