

ANILINE and o-TOLUIDINE in urine

8317

(1) Aniline: C ₆ H ₇ N	MW: 93.13	CAS: 62-53-3	RTECS: BW6650000
(2) o-Toluidine: C ₇ H ₉ N	MW: 107.16	CAS: 95-53-4	RTECS: XU2975000

METHOD: 8317, Issue 1

EVALUATION: PARTIAL

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BIOLOGICAL INDICATOR OF: exposure to (1) aniline and (2) o-toluidine

ACGIH BEI: none

SYNONYMS: (1) aniline: benzeneamine; aminobenzene; phenylamine
 (2) o-toluidine: 2-aminotoluene

SAMPLING	MEASUREMENT										
<p>SPECIMEN: Urine</p> <p>VOLUME: At least 4 mL of sample</p> <p>PRESERVATIVE: 5 g citric acid</p> <p>SHIPMENT: Freeze urine; ship on dry ice in an insulated container</p> <p>SAMPLE STABILITY: Sample stable for over 6 months at -65 °C</p> <p>CONTROLS: Collect urine from non-occupationally exposed workers</p>	<p>TECHNIQUE: HPLC with electrochemical detection</p> <p>ANALYTE: Aniline and o-toluidine</p> <p>TREATMENT: Base hydrolysis and liquid-liquid extraction</p> <p>INJECTION VOL.: 50 µL</p> <p>MOBILE PHASE: 37:53 methanol-phosphate buffer (pH 3.3), containing ~67 mg/L sodium dodecyl sulfate.</p> <p>FLOW RATE: 0.8 mL/min</p> <p>GUARD CELL: 1000 mV</p> <p>DETECTOR: Dual electrode coulometric electrochemical monitored at 600 mV.</p> <p>COLUMN: Highly endcapped C₁₈-RP column (Waters NovaPak), 300-mm x 4.6-mm, heated to 30 °C with 0.2 µm inline filter and guard column of same sorbent.</p> <p>CALIBRATION: Analyte in mobile phase.</p> <p>QUALITY CONTROL: Analyte in urine at 0, 4, 20, 100 ng/mL.</p> <p>RANGE: 1.4 to 1200 ng/mL</p> <p>ESTIMATED LOD: Aniline 1.4 ng/mL o-Toluidine 0.6 ng/mL</p> <p>PRECISION (S_r):</p> <table style="margin-left: 20px; border: none;"> <tr> <td>Aniline</td> <td>0.17 [4]</td> </tr> <tr> <td>Acetanilide</td> <td>0.16 @ 18 ng/mL</td> </tr> <tr> <td>o-Toluidine</td> <td>0.20</td> </tr> <tr> <td>n-Acetyltoluidine</td> <td>0.17 @ 16 ng/mL;</td> </tr> <tr> <td></td> <td>0.10 @ 200 ng/mL</td> </tr> </table>	Aniline	0.17 [4]	Acetanilide	0.16 @ 18 ng/mL	o-Toluidine	0.20	n-Acetyltoluidine	0.17 @ 16 ng/mL;		0.10 @ 200 ng/mL
Aniline	0.17 [4]										
Acetanilide	0.16 @ 18 ng/mL										
o-Toluidine	0.20										
n-Acetyltoluidine	0.17 @ 16 ng/mL;										
	0.10 @ 200 ng/mL										

APPLICABILITY: This method, monitors the parent compounds and their acetyl metabolites to remove the ambiguity from the aminophenol's origin.

INTERFERENCES: No interferences were observed for aniline or o-toluidine

OTHER METHODS: El Bayoumy developed a biological monitoring method for aniline and o-toluidine in 1986 [3], in which each liter of sample spiked with internal standard was lyophilized, reconstituted, concentrated, liquid/liquid extracted 3 times, evaporated to dryness, reflux hydrolyzed for 2.5 hrs, liquid/liquid again extracted 3 times, evaporated to dryness, reacted with pentafluoropropionic acid, before being analyzed by GC-ECD.

REAGENTS:

1. Aniline and o-toluidine stock solution, 100 mg/L, is prepared by dissolving 100 mg o-toluidine and 139 mg of aniline hydrochloride in 1 L of 0.1 N HCl.
2. Aniline and o-toluidine calibration solution, 1000 µg/L. Dilute 1.000 mL of the stock solution with water to 100 mL to produce a 1000 µg/L solution for spiking.
3. Aniline and o-toluidine calibration solution, 100 µg/L. Dilute 1.000 mL of the above 1000 µg/L calibration solution with water to 100 mL to produce a 100 µg/L solution. These standard solutions are stable at 4 °C for at least two weeks.
4. Mobile phase: Add 23.0 g of NaH₂PO₄•H₂O and 6 mL of 8.5 % phosphoric acid to a 2-L volumetric flask. Bring to near volume with water, mix, and adjust pH to 3.3 ± 0.05 with 8.5 % phosphoric acid or 50% sodium hydroxide, then bring to 2L mark. Add 1226 mL of methanol and 200 ± 0.5 mg of sodium dodecyl sulfate to a 4-L HPLC reservoir and mix. Blend the aqueous buffer with the methanol solution to prepare the mobile phase. Total liquid volume will shrink when liquids are mixed.
5. Water, highly purified. >10 MΩ.
6. Methanol, HPLC grade.
7. Sodium dihydrogen phosphate monohydrate, NaH₂PO₄•H₂O.
8. Sodium dodecyl sulfate.
9. Aniline hydrochloride.*
10. o-toluidine.*
11. Phosphoric acid 85%.*
12. Butyl chloride, HPLC grade.*
13. Sodium hydroxide, reagent grade pellets.*
14. Hydrochloric acid 0.1 N, Fisher certified.*
15. Citric acid, anhydrous, reagents grade.
16. Urine from non-occupationally exposed persons, who have no exposure to tobacco smoke.*

* See SPECIAL PRECAUTIONS

EQUIPMENT:

1. Bottles, polypropylene, screw-top, 8-oz, 250-mL.
2. Bottles, polypropylene, screw-top, 2-oz, 60-mL.
3. HPLC system consisting of the following components in series; 5-L reservoir, in-line degasser filter, pump, pulse dampener, electrochemical guard cell (1.0 V), auto injector, in-line 0.2 µm in-line filter, a guard column, a 300 mm X 4.6 mm analytical column in an oven set at 30 °C, dual electrode coulometric electrochemical detector connected to a data acquisition system, and a liquid waste container. The column packing material is highly end-capped 3 µm C₁₈-RP particles (Waters NovaPak or equivalent). The detector has two electrodes, one of which is used to remove potential interferences oxidizing below 400 mV and the other for analyte detection at 600 mV.
4. pH meter.
5. Vortex mixer.
6. Rotatory mixer.
7. Centrifuge tubes, 15-mL with teflon lined caps, disposable.
8. Reagent dispenser, 8-mL.
9. Auto-injector vials.
10. Water bath at 80 °C.
11. Dispensing pipette, 2-mL and 5-mL, disposable.
12. Micropipette, 1000-µL
13. Pasteur transfer pipettes.
14. Volumetric pipettes *, 1-, 2-, 3-, 4-, 5-, and 10-mL.
15. Syringes, 3-cc plastic disposable.
16. Syringe filters, Anotop™ 10, 0.2 µm pore size.
17. Volumetric flasks *, convenient sizes for preparing standard solutions.
18. Scintillation vials, 10-mL polypropylene.

* Wash all permanent glassware with 0.1 N NaOH, 0.1 N HCl, water, and then methanol before use.

SPECIAL PRECAUTIONS: Urine samples may contain a number of bacterial and viral agents, including hepatitis B virus, and should be handled using Biosafety Level 2 practices, containment equipment, and facilities. [CDC & NIH, *Biosafety in Microbiological and Biomedical Laboratories*, 3rd ed., HHS Publication No. (CDC) 93-8395 (1993)]. Methanol and butyl chloride are NFPA level 3 fire hazards. Concentrated phosphoric acid and sodium hydroxide are a NFPA level 3 health and level 2 reactivity hazards, besides being corrosive. o-Toluidine is a bladder carcinogen, and may cause damage to the blood or kidneys. Aniline may be fatal if swallowed, inhaled, or absorbed through the skin. Aniline causes irritation to skin, eyes, and respiratory tract, and may cause cyanosis.

SAMPLING:

1. Timing of Collection: In humans, aniline is rapidly metabolized and excreted in the urine [1]. Rat data suggest that o-toluidine likewise is rapidly metabolized and excreted [2]. Thus, biological monitoring using pre and post shift urine samples is recommended in order to capture the metabolites quickly after exposure.
2. Collect urine in one or more 250-mL polypropylene bottles. Measure the total volume and transfer approximately 50 mL to a 60-mL polypropylene bottle containing 5.0 g of citric acid preservative. Label the bottle with the code unique to that specimen. Freeze immediately on dry ice.
3. Ship in a packing container that is designed for dry ice storage and transport. Store at -65 °C.

SAMPLE PREPARATION:

4. With each batch of 20 field urine samples, also process 2 quality control samples, 2 water blanks, and 2 field samples analyzed in a previous batch, a total of 26 samples per batch.
5. Assemble 26 conical centrifuge tubes in a racks.
6. Add 1.00 ± 0.05 g of NaOH pellets, cap, and label tubes.
7. Thaw samples to room temperature. To minimize analyte loss, keep caps tightly sealed and minimize time between steps 7-14.
NOTE: Frozen urine samples are inhomogeneous. Insure that the samples are completely thawed and well mixed before removing aliquot.
8. Add 4.0 mL of sample to each tube and recap tightly.
9. Heat for 2 hr ± 5 minutes at 80 °C in a water bath.
10. Cool to room temperature, add 8.0 mL of butyl chloride to tubes and recap.
11. Tumble tubes for 10 minutes at 50 rpm, and centrifuge for 5 minutes at 3000 rpm.
12. Transfer 5.0 mL of the upper butyl chloride layer to a second set of labeled centrifuge tubes.
13. Add 1.0 mL of 0.1 N HCl to tubes containing butyl chloride solution.
14. Tumble tubes for 10 minutes at 50 rpm, and centrifuge for 5 minutes at 3000 rpm.
15. Remove lower aqueous layer with a Pasteur pipette, and transfer to a 3 cc syringe barrel fitted with a 10-mm 0.2 µm filter.
16. Insert syringe plunger and inject the aqueous extract through the filter into a HPLC auto-injector vial and seal.
17. Order the samples and standard solutions in the auto-injector carousel in a fixed pattern of two standard solutions bracketing every two sample extracts, e.g. standard, unknown, unknown, standard, unknown, standard. Within the fixed order randomize the standards and samples separately.
18. Analyze samples by HPLC (Steps 29 to 32).

CALIBRATION AND QUALITY CONTROL:

19. Label ten 50-mL volumetric flasks with the concentrations listed in the second column of the table below. Using the table below, prepare the standard solutions listed by diluting the indicated volume of stock standard solution to 50 mL with mobile phase.

	Calibration Solution (µg/L)	Volume of Stock Standard Solution (mL)		Volume of Mobile Phase (mL)
		1000 µg/L	100 µg/L	
1.	100	5.0		45.0
2.	80	4.0		46.0
3.	60	3.0		47.0
4.	40	2.0		48.0
5.	20	1.0		49.0
6.	10		5.0	45.0
7.	8		4.0	46.0
8.	6		3.0	47.0
9.	4		2.0	48.0
10.	2		1.0	49.0

The calibration solutions are stable at 4 °C for at least 2 weeks. It is recommended to, prepare half of the calibration solutions fresh with each set of samples. Alternate the use of duplicate primary stock standard solutions so that every other set of calibration solutions is made from the alternate stock standard solutions.

20. Analyze these 10 calibration solutions with each batch of samples, so that every 2 field samples are bracketed with calibration solutions.
21. Because the calibration results are non-linear at low concentrations a quadratic curve should be prepared using data from the calibration solutions. Make the nominal mass of amine in µg injected the independent X variable and its peak height response the dependent variable of the calibration curve.

$$Y = aX^2 + bX + c$$

where a, b, and c are regression coefficients

22. Also, prepare quality control samples by collecting 1 L of fresh urine from unexposed non-smoking individuals, who are not taking medication.
23. To one liter of this fresh urine add 100 g of citric acid.
24. Add 25, 5, and 1 mL of stock 1000-µg/L standard solution to three 250-mL volumetric flasks, and dilute to the mark with the acidified urine. The nominal concentrations of these samples, i.e. 100, 20, and 4 µg/L, respectively, must be corrected for the average concentration of o-toluidine and aniline in the unfortified urine.
25. After each standard solution is added to urine, the fortified and unfortified urine is aliquoted into 10-mL polypropylene scintillation vials and stored at -65 °C.
26. Analyze these quality control samples with each batch and maintain quality control charts.
27. Also, analyze at least one replicate sample from a previous batch with each new batch to assess precision with variable matrices.
28. Blind field splits are also recommended as a check on method precision.

MEASUREMENT:

29. Set the chromatograph according to manufacturer's recommendations and to the conditions given on page 1. Inject 50 µL of final solution in step 16.
30. Measure peak heights of aniline and o-toluidine in the calibration solutions and in the samples.

31. Peak purity can be confirmed, if necessary, by re-analyzing the extract and three calibration solutions, once with Electrode 2 set at 520 mV and once at 600 mV. The response ratios of the unknown at these two voltages should match the response ratio of the calibration solutions within ± 3 standard deviations.
32. If the sample gives a peak for aniline and o-toluidine with a peak height out of the calibration range, re-analyze the extract using a 5 μL injection. If the 5 μL injection still gives a peak out of the calibration range, reduce the detector gain.

CALCULATIONS:

33. Calculate the concentration, C(pg/ μL), of aniline or o-toluidine in the unknown from the peak heights and the calibration curve for aniline or o-toluidine, respectively (C, $\mu\text{g/L}$).

$$C = -b + \frac{\sqrt{b^2 - 4a(c - Y)}}{2a} \cdot \frac{1}{D} \cdot \frac{E}{F} \cdot \frac{G}{H}, \text{pg} / \mu\text{L}$$

a, b, c, and Y are defined in step 21

D = μL of injection volume

E = initial volume of urine

F = volume of 0.1 M HCl back extractant

G = initial volume of butyl chloride

H = transferred volume of butyl chloride

This equation does not account for a change in detector gain.

GUIDES TO INTERPRETATION:

This method was applied to 171 urine specimens from a chemical plant worker population with a known excess of bladder cancer. The median levels of o-toluidine were: exposed preshift = 11 $\mu\text{g/L}$; exposed postshift = 65 $\mu\text{g/L}$; nonexposed preshift = 0.7 $\mu\text{g/L}$; and nonexposed postshift = 2.6 $\mu\text{g/L}$. The median levels of aniline were: exposed preshift = 11 $\mu\text{g/L}$; exposed postshift = 23 $\mu\text{g/L}$; nonexposed preshift = 2.0 $\mu\text{g/L}$; and nonexposed postshift = 3.2 $\mu\text{g/L}$. [4-8]

The Karam El-Bayoumy et al. study [3] reported the total mass of aniline and o-toluidine excreted by urine of 19 occupationally exposed subjects, and they found that the workers excreted from 0.02 to 8.8 μg of aniline and 0.3 to 12.9 μg of o-toluidine during a 24-hour period.

EVALUATION OF METHOD:

The American Conference of Governmental Industrial Hygienists has recommended a biological exposure index (BEI) for aniline of 50 mg of 4-aminophenol per milligram of creatinine, measured in end-of-shift urine specimens. 4-Aminophenol is the ring hydroxylated metabolite of aniline [1]. Before a BEI for o-toluidine can be established, a relationship between exposure to o-toluidine and excretion of the metabolite must be established. Rat studies suggest that o-toluidine is also substantially metabolized by a ring hydroxylation pathway, but no one has demonstrated the presence of the metabolite in the urine of humans exposed to o-toluidine [2]. Other xenobiotics, i.e., acetaminophen, reach the same metabolic fate of 4-aminophenol as does aniline. This method, monitors the parent compounds and their acetyl metabolites to remove the ambiguity from the aminophenol's origin.

The method was characterized during a field study that included 45 batches of samples. Each batch contained QC samples of aniline with nominal levels of aniline spiked at 6.9 ng/mL, 18 ng/mL, and 77 ng/mL and with nominal levels of o-toluidine spiked at 4.2 ng/mL, 20 ng/mL, and 102 ng/mL. QCs samples were also made using acetanilide and N-acetyl-o-toluidine spiked to an equivalent free amine concentration of 19 ng/mL and 16 ng/mL, aniline and o-toluidine. The precision and recovery data reported on page 8317-1 was an average of

these levels, and reported below in a more detailed results table. In addition, blind field splits were also used to characterize the precision of the method.

RECOVERY [4]	aniline	109 % @ 6.9 ng/mL
	aniline	97 % @ 18 ng/mL
	aniline	93 % @ 77 ng/mL
	acetanilide	96 % @ 19 ng/mL
	o-toluidine	101 % @ 4.2 ng/mL
	o-toluidine	93 % @ 20 ng/mL
	o-toluidine	86 % @ 102 ng/mL
	N-acetyltoluidine	83 % @ 16 ng/mL
PRECISION (\bar{S}_r) [4]	aniline	0.26 @ 6.9 ng/mL
	aniline	0.14 @ 18 ng/mL
	aniline	0.12 @ 77 ng/mL
	acetanilide	0.16 @ 19 ng/mL
	o-toluidine	0.32 @ 4.2 ng/mL
	o-toluidine	0.17 @ 20 ng/mL
	o-toluidine	0.14 @ 102 ng/mL
	N-acetyltoluidine	0.17 @ 16 ng/mL
	Duplicate blind splits	
	Pairs of duplicates	43
	Range (ng/mL)	10 - 350
	Average Relative Standard Deviation	23 %

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METHOD DEVELOPED BY:

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