HIPPURIC and METHYL HIPPURIC ACIDS in urine 8301

(1) hippuric acid	$C_6H_5CONHCH_2CO_2H$	FW:	179.18	CAS:	495-69-2	RTECS: MR815000
(2) 2-methyl hippuric acid	$CH_3C_6H_5CONHCH_2CO_2H$		193.20		42013-20-7	
(3) 3-methyl hippuric acid	$CH_3C_6H_5CONHCH_2CO_2H$		193.20		27115-49-7	
(4) 4-methyl hippuric acid	$CH_3C_6H_5CONHCH_2CO_2H$		193.20		27115-50-7	

METHOD: 8301, Issue 3 **EVALUATION: PARTIAL** **Issue 1: 15 February 1984**
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BIOLOGICAL INDICATOR OF: Exposure to toluene and xylenes

SYNONYMS: (1) hippuric acid: N-benzoylglycine
(2) 2-methyl hippuric acid: N-(o-Toluoyl) glycine
(3) 3-methyl hippuric acid: N-(m-Toluoyl) glycine
(4) 4-methyl hippuric acid: N-(p-Toluoyl) glycine

SAMPLING		MEASUREMENT	
SPECIMENS:	1. Pre-shift urine 2. End of shift urine after 2 days exposure	**TECHNIQUE:**	HPLC-UV DETECTION
VOLUME:	Complete spot void	**ANALYTES:**	(1) hippuric acid (2) 2-methyl hippuric acid (3) 3- & 4-methyl hippuric acids
PRESERVATIVE:	A few crystals of thymol; store @ 4°C	**EXTRACTANT:**	Ethyl acetate
SHIPMENT:	Pack in styrofoam shipper with bagged refrigerant, ship overnight	**INJECTION VOLUME:**	10 µL
		WAVELENGTH:	254 nm
SAMPLE STABILITY:	Stable 30 days @ 4°C	**COLUMN:**	Reverse phase (C18), run at 37°C
CONTROLS:	Collect samples from unexposed, matched population.	**MOBILE PHASE:**	84/16/0.025% (v / v / v) water / acetonitrile / glacial acetic acid; 1.5 mL/min
		CALIBRATION:	Synthetic urine solutions of analyte.
		RANGE:	10.0 to 1000 µg/mL.
		ESTIMATED LOD:	(1) 4 µg/mL (2) 5 µg/mL (3) 6 µg/mL NOTE: See Method Evalution
		PRECISION ($\bar{S}_r$):	(1) 0.020 (see Table 1) (2) 0.015 (see Table 1) (3) 0.011 (see Table 1)

APPLICABILITY: Hippuric acid and methyl hippuric acids are the principal metabolites of toluene and xylene, respectively. An occupational exposure to either of these organic solvents may be monitored by following the pattern of excretion of these metabolites in urine. Due to overlap in the range of hippuric acid concentration between exposed workers and non-exposed workers, monitoring urinary hippuric acid concentrations are better suited for groups of workers than for individuals [2].

INTERFERENCES: None known; however, para- and meta- isomers elute together in this system. There are other sources of hippuric acid such as food preservatives, ethyl-benzene, and styrene.

OTHER METHODS: This is based on the method of Matsui, et al [3]. Method 8300 can be used for screening. Isotachophoresis has also been used [4]. More recent HPLC methodologies provide better resolution for these compounds [5].

REAGENTS:

1. Thymol, USP.
2. Sodium chloride.
3. Hydrochloric acid (6N).*
4. Ethyl acetate, HPLC grade.
5. Hippuric acid, reagent grade.
6. 2-Methyl hippuric acid, reagent grade.
7. 3-Methyl hippuric acid, reagent grade.
8. 4-Methyl hippuric acid, reagent grade.
9. Combined hippuric acids stock solution, 1 mg/mL per analyte. Add 35 mg each of hippuric acid, 2-methyl hippuric acid, 3-methyl hippuric acid, and 4-methyl hippuric acid to 35 mL synthetic urine in a 40-mL vial. Cap tightly and sonicate for 30 min, then place in 30°C water bath for 5 min. Visually inspect to verify that all compounds are in solution.
   NOTE: The hippuric acid solution will precipitate out at room temperature, so this preparation should be used immediately after removal from the 30°C water bath.
10. Mobile phase. Add 840 mL distilled water to 160 mL acetonitrile (HPLC grade) and 250 µL of glacial acetic acid. Mix and filter.
11. Pre-purified nitrogen, 99.9%.
12. Synthetic urine (Uri sub™ from CST Technologies, Inc., Great Neck, NY).
13. Pooled, unexposed, human urine [1].

* See SPECIAL PRECAUTIONS

EQUIPMENT:

1. Bottles, polyethylene, 250-mL.
2. Refrigerant, bagged ("Blue Ice," or equivalent).
3. HPLC system consisting of sample injector, pump, ultraviolet detector at 254 nm, strip chart recorder, integrator, RP C-18 column, and column heater (Supelco Discovery #322201-01 or equivalent).
4. Heated water bath purged by nitrogen blow-down apparatus.
5. Centrifuge.
6. Analytical balance.
7. Tubes, 15-mL borosilicate glass with caps.
8. HPLC vials and caps with limited volume inserts (Kimble Glass, Inc., Art. No. 60745N-1232).
9. Micro-syringes: 10-µL, 100-µL, 250-µL, 500-µL, 1-mL and 10-mL.
10. Positive displacement pipette (40-µL).
11. Rotation Mixer (Fisher model 34601 or equivalent).

SPECIAL PRECAUTIONS: Samples of urine collected from humans pose a real health risk to laboratory workers who collect and handle these samples. These risks are primarily due to personal contact with infective biological samples and can have serious health consequences, such as infectious hepatitis, and other diseases. Pre-immunization against Hepatitis C is highly recommended. Those who handle urine specimens should wear protective gloves, and avoid aerosolization of the samples. Mouth pipetting, of course, must be avoided.
Acids are extremely corrosive; work with them in a fume hood and wear protective safety equipment.

SAMPLING:

1. Collect pre-shift and post-shift urine in a 250-mL polyethylene bottle containing a few crystals of thymol.
   NOTE: Take the sample at the end of the second day of suspected exposure to toluene or xylene. Also take pre-exposure samples and samples from non-exposed workers as controls.
2. Pack bottles in styrofoam shipper with bagged refrigerant and ship overnight.

SAMPLE PREPARATION:

3. Perform a creatinine determination on an aliquot of urine [6].
4. Pipette 1.0 mL of well-mixed urine into a 15-mL borosilicate glass tube with cap.
5. Add 80 µL of 6 N Hcl with positive displacement pipette, mix, and add 0.3 grams sodium chloride.

6. Add 4 mL ethyl acetate. Mix for 2 minutes by rotation.
7. Centrifuge at 100 x gravity for 5 minutes.

NOTE: $RPM = 10^3 \sqrt{RCF / 1.12 r_{max}}$

r_{max} = rotor radius
Relative Centrifugal Force (RCF) = gravity

8. Transfer 200 µL of the organic (upper) layer to an HPLC vial and evaporate to dryness (~ 30 minutes) using a heated (~30°C) water bath and a gentle stream of nitrogen.
9. Re-dissolve the residue in 200 µL of distilled water.

CALIBRATION AND QUALITY CONTROL:

10. Prepare working standards over the range of 10 to 1000 µg/mL by dilution of combined hippuric acids stock solution using synthetic urine. Working standards are stable for 1 week at room temperature and stable for 30 days at 4°C [1].
11. Include control samples from an unexposed, matched population with each analytical run. If controls were not sent, use pooled, unexposed human urine.
12. Extract and analyze the working standards together with samples, and controls (steps 4 through 9, 14 and 15).
13. Analyze a duplicate field sample injection at a frequency of one replicate per 10 samples to monitor instrument accuracy.

MEASUREMENT:

14. Set up the HPLC according to the manufacturer's recommendations and to conditions on page 8301-1.
15. Inject 10 µL of the standards, samples, and control samples into the HPLC. Determine peak heights.

CALCULATIONS:

16. Calculate the concentration of each analyte in the urine sample, C_u (µg/mL), using the standard curve to correlate the peak height with the concentration amount.
17. Calculate the grams of analyte per grams of creatinine in the urine sample, C (g/g creatinine), using the concentration of analyte in the urine sample, C_u (µg/mL), and the concentration of creatinine in urine determined in step 3, C_R (g creatinine/L urine).

$$C = \frac{C_U}{C_R} \cdot \frac{1000\,\mu L}{L}$$

GUIDES TO INTERPRETATION:

1. Typical excretion of hippuric acid in non-exposed individuals is 1.0 g/g creatinine [2]. Methyl hippuric acids are not found in non-exposed humans.
2. The Threshold Limit Value (TLV®) [9] for urine samples collected at the end of a work shift is 1.6 g/g creatinine for hippuric acid and 1.5 g/g creatinine for methyl hippuric acids.

EVALUATION OF METHOD:

LOD/LOQ studies were performed using standards in synthetic urine, standards in pooled urine, and standards in water. Standards dissolved in synthetic urine gave LODs of 4 µg/mL, 5 µg/mL, 6 µg/mL for hippuric acid, 2-methyl hippuric acid, 3-methyl hippuric acid, and 4-methyl hippuric acid, respectively. Water proved to be a poor matrix for hippuric acids, giving a high variability. Pooled urine also gave a high variability due to an initial background. Endogenous hippuric acids in pooled urine will skew the calibration curve.

Recovery study data shows greater than 96% overall recovery for all analytes from spiked synthetic urine. Overall recovery rates were slightly lower (93%) for spiked pooled urine, possibly due to variability in naturally occurring hippuric acids. Precision, Accuracy, and Bias data (Table 1) were obtained for synthetic urine using 7 replicates at 6 different concentration levels from 10 µg/mL to 1000 µg/L. Results using pooled urine were similar.

A 30-day stability study showed that the hippuric acids in synthetic urine were stable for 7 days at 22°C and 30 days at 4°C. In a single synthetic urine sample, hippuric acid was stable through temperature changes from -20°C to 30°C for 24 hours. Sonication did not affect hippuric acid stability in the samples.

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Previous Issues

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TABLE 1. OVERALL PRECISION, ACCURACY, AND BIAS

Compound	Range Studied (µg/mL)	Estimated Accuracy (%)	Bias		Overall Precision ($\hat{S}_{rT}$)	Instrument Precision (S_r)
			Average	Range		
Hippuric acid	10-1000	16.0	-0.081	-0.312-0.0237	0.0538	0.020
2-Methyl hippuric acid	10-1000	19.8	-0.099	-0.286-0.0141	0.0672	0.015
3-Methyl and 4-Methyl hippuric acids	10-1000	18.9	-0.097	-0.2959-0.3869	0.0615	0.011