SUPPLEMENTARY MATERIAL SECTION: ANALYTICAL CHEMISTRY

for

Comparison of Nicotine and Carcinogen Exposure with Water pipe and Cigarette Smoking

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Nicotine in Plasma

Concentrations of nicotine in plasma were determined using gas chromatography-mass spectrometry (GC-MS) [1], modified for analysis using a triple quadrupole mass spectrometer. This consisted of operating the mass spectrometer in the chemical ionization mode (isobutane reagent gas), and using selected reaction monitoring (*m/z* 163 to 84 for nicotine, and *m/z* 172 to 89 for the internal standard, nicotine-d_9) for quantitation. This modification provides a lower limit of quantitation of 0.2 ng/mL.

NNAL in Urine

NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1- butanol) is a carcinogen and a metabolite/biomarker of the potent lung carcinogen NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) [2]**.** Concentrations of total NNAL (NNAL plus its glucuronide) in urine were determined by a published method using liquid chromatography – tandem mass spectrometry

(LC-MS/MS) [3]. A brief description is as follows: the internal standard, NNAL-d_3 was added, and the samples were incubated with beta-glucuronidase enzyme to cleave the conjugates for determination of total NNAL. The analyte was extracted using a liquid/liquid extraction procedure, and converted to the hexanoate ester derivative. Following chromatography using a gradient elution, the analyte was quantitated using electrospray ionization (ESI) and selected reaction monitoring (SRM). The lower limit of quantitation (LLOQ) is 0.25 pg/mL (0.0012 pmol/mL).

PAH Metabolites in Urine

PAHs (polycyclic aromatic hydrocarbons) are a major class of carcinogens formed by incomplete combustion. PAH metabolites were also determined using LC-MS/MS. [4] Briefly, stable isotope-labeled internals standards were added, and the samples were incubated with beta-glucuronidase enzyme to cleave the conjugates. Following a liquid/liquid extraction, the analytes were converted to pentafluorobenzyl derivatives. The analytes are separated using a gradient elution, and quantitated using electron capture atmospheric pressure chemical ionization (ECAPCI) and SRM. The LLOQ for 2-naphthol is 0.25 ng/mL; the LLOQs for the other analytes are 0.025 ng/mL.

Mercapturic Acid Metabolites of VOCs

Tobacco smoke contains carcinogenic volatile organic compounds (VOCs), and many of these are metabolized by conjugation with glutathione and excretion as mercapturic acids, which can be useful biomarkers. Mercapturic acid metabolites of VOCs were determined by LC-MS/MS using electron capture atmospheric pressure chemical ionization (ECAPCI), using the same principle to enhance detection as described in our PAH method described above.

Instrumentation. LC-MS/MS analyses were carried out with an Agilent (Palo Alto, CA) 1200 HPLC interfaced to a Thermo-Finnigan (San Jose, CA) TSQ Quantum Ultra triple-stage quadrupole mass spectrometer, with an atmospheric pressure chemical ionization (APCI) source.

Standards and Reagents. Mercapturic acid standards and deuterium-labeled internal standards were obtained from Toronto Research Chemicals, North York, Ontario, Canada, or were synthesized in our laboratory. HPLC grade methanol and water from Honeywell/Burdick and Jackson (Muskegon, MI) were used to prepare the LC mobile phase. Methylene chloride, isopropyl alcohol, ethyl acetate, and pentane used for extractions were HPLC or Optima grade from Fisher Scientific (Fair Lawn, New Jersey). Pentafluorobenzyl bromide and N,N-diisopropylethylamine were obtained from Aldrich Chemical Company (Milwaukee, WI).

*Preparation of Standards and Controls***.** Standards were prepared by spiking "artificial urine" [4] with the analytes at ten concentrations spanning the expected range. Pooled urine from 3 non-smokers was used to prepare quality control samples. Standards and controls were stored frozen at -20°C until use.

Sample Preparation. Deuterium-labeled internal standards in 50 µL water were added to 1 mL standards in artificial urine or to 1 mL urine samples followed by 0.9 mL of saturated potassium bromide and 0.1 mL saturated potassium hydrogen sulfate. They were then extracted with a mixture of methylene chloride, isopropyl alcohol, and ethyl acetate (1:1:1, 3 mL). The extracts were evaporated using a stream of nitrogen at 60° C, and the analytes were converted to pentafluorobenzyl ester derivatives by treatment with 120 µL acetonitrile, 15 µL 10% w/v pentafluorobenzyl bromide (caution, lacrimator!) in acetonitrile and 15 µL N,N-diisopropylethylamine at 60° C for 30 min as described by Tsikas [5]. Following derivatization, the tubes were dried in a stream of nitrogen to remove excess derivatizing agent, cooled, and 0.25 mL of saturated aqueous potassium dihydrogen phosphate was added. The analytes were extracted with 2 mL of 50:50 (v/v) pentane/methylene chloride. The extracts were evaporated to dryness using a stream of nitrogen, and the analytes were dissolved in 0.15 mL of methanol for LC-MS/MS analysis.

Liquid Chromatography. The extracts (10 µL) were chromatographed with a gradient elution (aqueous methanol to 100% methanol) on a Phenomenex Synergi Polar-RP column (150 mm x 4.6 mm, 4 micron) with a flow rate of 0.7 mL/minute. The initial composition was 20% methanol, held for 1 min, which was then changed to 60% methanol over 0.1 min. This

composition was held from 1.1 to 15.9 min post-injection, and then changed to 100% methanol over 0.6 min. 100% Methanol was maintained from 16.5 min to 22 min post-injection and then returned to 20% methanol to re-equilibrate the column for the next injection. *Note*: A Supelco HSF5 column (150 mm x 4 mm, 5 micron) can be used as an alternative to give better separation of 2- and 3- hydroxypropylmercapturic acid.

Mass Spectrometry. The mass spectrometer was operated using APCI in the negative ion mode (ECAPCI). The ion source parameters were optimized by infusing a solution of the pentafluorobenzyl ester derivative of MHBMA into the ion source *via* a syringe pump. The vaporizer temperature was 450° C, the heated capillary temperature was 225° C, and the corona discharge current was 50 μA. Data was acquired in the selected reaction monitoring (SRM) mode. The SRM transitions and collision energies for the analytes and internal standards are given in Table S-1. The mass resolution (FWHM) was set at 0.3 amu for Q1 and 0.7 for Q3, with the exception of PMA, for which Q1 was set at 0.1 amu, and Q3 was set at 0.7 amu. Representative chromatograms are shown in Figure S-1.

Data Analysis. The Finnigan XCalibur/LC Quan software was used to generate calibration curves (linear regression, 1/*X* weighting) and calculate concentrations using peak area ratios of analyte/internal standard (Table S-2). Calibration curves for the challenging analytes MHBMA and PMA are shown in Figures S-2 and S-3.

Validation. The method was evaluated for precision, accuracy, and lower limit of quantitation by replicate analysis of spiked non-smokers' urine and artificial urine samples, at concentrations spanning the expected concentration ranges (Table S-3) as described by Shah et al [6] and Viswanathan et al [7].

Results and Discussion. Conversion of the analytes to the pentafluorobenzyl ester derivatives and mass spectrometric analysis using ECAPCI provided high sensitivity, and for most analytes clean chromatograms (Figure S-1) and low limits of quantitation (Table S-3) were obtained. Precision and accuracy met the requirements for bioanalytical methods used in drug development studies [6, 7] (Table S-3). Concentrations and 24-hour urinary excretion for the 13 subjects are

presented in Table 4, along with published data for cigarette smokers. Concentrations and excretion in our subjects were in the ranges previously reported, but in general somewhat lower. Likewise, concentrations of PAH metabolites were similar but somewhat lower than previously reported for cigarette smokers (Table S-5). This may be due to our subject population, which were water pipe smokers who also smoke cigarettes, and who smoked on average fewer cigarettes per day than the average U.S. cigarette-only smoker. The study was carried out on a research ward, which might also have affected smoking intensity.

The butadiene metabolite MHBMA deserves further discussion. It exists as multiple isomers. Most past studies have measured a mixture of two isomers, MHBMA-1 = N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine and MHBMA-2 = N-Acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine. Pure standards have generally not been available, and most investigators have used a mixture of these two and reported a sum of their concentrations. In our study, we reported concentrations of one of the two isomers. A recent publication reported that a third isomer, MHBMA-3 = N-Acetyl-S-(4-hydroxy-2-butenyl)-L-cysteine, is present in urine at higher concentrations than either MHBMA-1or MHBMA-2 [8]. In addition, this publication reported concentrations for all three isomers separately, as standards had been custom synthesized.

References

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Table S-1. SRM Transitions and Collision Energies

Analyte[a]	Parent Ion	Product Ion	Collision Energy (eV)
HEMA	206	77	16
CNEMA	215	162	16
3-HPMA	220	91	17
2-HPMA	220	91	17
MHBMA	232	103	16
AAMA	233	104	18
PMA	238	109	18
HEMA-Acetyl-d_3	209	77	18
CNEMA-Acetyl-d_3	218	165	18
3-HPMA-Acetyl-d_4	224	91	18
2-HPMA-Acetyl-d_3	223	91	18
MHBMA-Acetyl-d_3	235	103	18
AAMA-Acetyl-d_3	236	104	18
PMA-Acetyl-d_3	241	109	18

[a] Abbreviations: HEMA = 2-Hydroxyethylmercapturic acid; CNEMA = 2-Cyanoethylmercapturic acid; 3-HPMA = 3-Hydroxypropylmercapturic acid; 2-HPMA = 2-Hydroxypropylmercapturic acid; MHBMA = 2-Hydroxy-3-buten-1-yl-mercapturic acid or isomer(s); AAMA = 2-Carbamoylethylmercapturic acid; PMA = Phenylmercapturic acid.

Table S-2. Equations for Mercapturic Acid Calibration Curves

Analyte[a]	Concentration Range, ng/mL	Regression Equation	R^2
HEMA	LOQ - 100	Y = 0.00377+0.176*X	0.9991
CNEMA	LOQ - 200	Y = 0.00160+0.0892*X	0.9992
3-HPMA	LOQ - 1000	Y = 0.000473+0.00455*X	0.9993

2-HPMA	LOQ - 1000	Y = 0.0175+0.0965*X	0.9997
MHBMA	LOQ - 100	Y = 0.000183+0.0319*X	0.9989
AAMA	LOQ - 1000	Y = 0.00142+0.00413*X	0.9995
PMA	LOQ - 20	Y = -0.000922+0.126*X	0.9997

[a] See Table S-1 for abbreviations

Table S-3. Precision and Accuracy for Mercapturic Acids in Urine, Intra-day (N=6)

Analyte[a]	Added Amount (ng/mL)	Expected Amount[b] (ng/mL)	Measured Mean (ng/mL)	Accuracy (Percent of Expected)	Precision CV (%)
HEMA	0		1.01		10.6
	2	3	3	100	5
	5	6	6.03	100	6
	20	21	20.1	96	6
	40	41	40	98	6
LOQ[c] (Artificial Urine)	0.2				
CNEMA	0		0.55		12.4
	2	2.55	2.39	94	11
	5	5.55	5.93	107	3
	20	20.6	18	88	3
	500	501	434	87	1
LOQ[c] (Artificial Urine)	0.5				
3-HPMA	0		101		1.6
	100	201	192	95	2
	250	351	338	96	3
	1000	1101	1016	92	2
	4000	4101	3878	95	3
LOQ[c] (Artificial Urine)	1		101		
2-HPMA	0		7.4		5.8
	10	17.4	16.5	95	6
	25	32.4	30.9	96	8
	100	107.4	104	96	3
	200	207.4	202	98	5
LOQ[c] (Artificial Urine)	1				
MHBMA	0		0.35		3
	1	1.35	1.13	84	8
	2.5	2.85	3.01	106	10
	10	10.35	10.8	105	8
	20	20.35	20.9	103	4
LOQ[c] (Artificial Urine)	0.2				
AAMA	0		25.7		5.8
	50	75.7	75.8	100	3
	125	150.7	153	101	4
	500	525.7	494	93.9	1
	1000	1025.7	950	92.7	4
LOQ[c] (Artificial Urine)	0.5				
PMA	0		0.05	BLQ	24
	0.2	0.25	0.25	99	10
	0.5	0.55	0.54	99	8
	2	2.05	1.97	96	3
	10	10.05	9.99	100	3
LOQ[c] (Artificial Urine)	0.1				

[a] See Table S-1 for abbreviations

[b] Added amount + mean amount measured in blank urine (pool of three non-smokers)

[c] The LOQ values listed are for spiked artificial urine, 6 replicates, which give a coefficient of variation <15%.

Table S-4. Concentrations of Mercapturic Acids in Urine of Cigarette Smokers. Comparison with Literature Values.

Biomarker[a]	This Study Mean, ±SD (ng/mL)	Ref [8] Mean, ±SD (ng/mL)	Ref [9] Median ng/mL	This Study Mean, ±SD (nmol/24 h)	Ref [10] Mean, ±SD (nmol/24 h)	Ref [11] Mean, ±SD (nmol/24 h)
HEMA	4.3±3.8	1.9±3.7		39±33	102±47	
CNEMA	45±35	187±181	240	414±273		
3-HPMA	340±219	1546±1643		3013±1411	10020±5150	
2-HPMA	69±60	185±235		580±426		
MHBMA[b]	0.70±0.4[c]	<LOD[d], 1.8±2.1[e]	<2[f]	6.0±2.5[c]	66.1±69.4[f]	371[f]
AAMA	71±42	196±180		623±258		
PMA	0.66±0.58	0.92±2.11		6.1±4.9	3.2±3.8	

[a] See Table S-1 for abbreviations

[b] MHBMA-1 = N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine; MHBMA-2 = N-Acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine. See reference [8].

[c] Either MHBMA-1 or MHBMA-2

[d] MHBMA-1

[e] MHBMA-2

[f] Measured as a mixture of MHBMA-1and MHBMA-2

Table S-5. Concentrations of PAH Metabolites in Urine of Smokers. Comparison with Literature Values.

Biomarker[a]	This Study Mean ng/L	Ref [12] Mean ng/L
2-Naph	6457	8597
2-Fluor	494	990
3-Fluor	268	592
1-Phen	49	193
2-Phen	123	88
1-HP	105	104

[a] Abbreviations: 2-Naphth = 2-Naphthol; 2-Fluor = 2-Hydroxyfluorene; 3-Fluor = 3-Hydroxyfluorene; 1-Phen = 1-Hydroxyphenanthrene; 2-Phen = 2-Hydroxyphenanthrene; 1-HP = 1-Hydroxypyrene

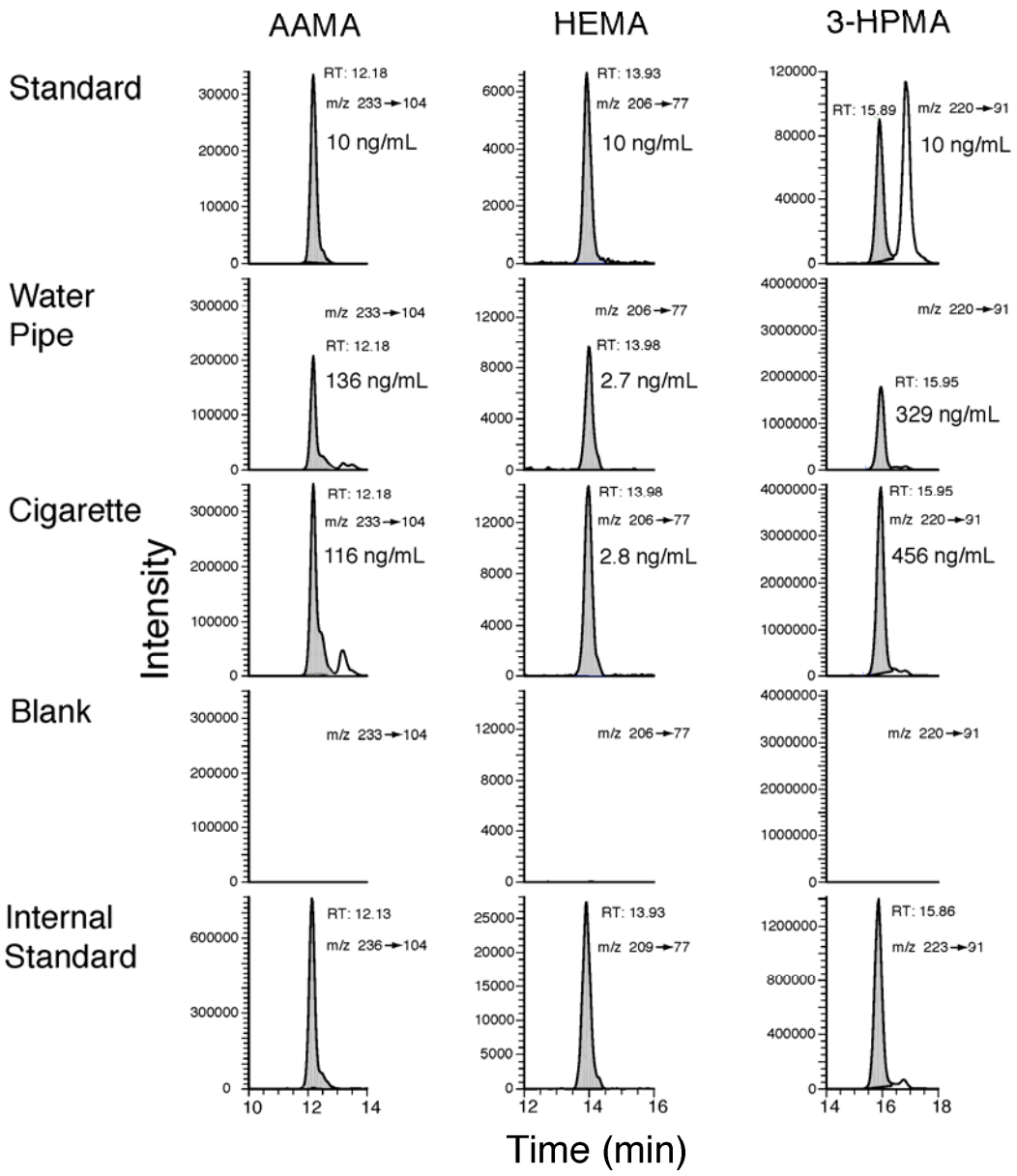

Figure S-1A. Chromatograms of urine extracts containing mercapturic acids: Metabolites of acrylamide (AAMA), ethylene oxide (HEMA), and acrolein (3-HPMA).

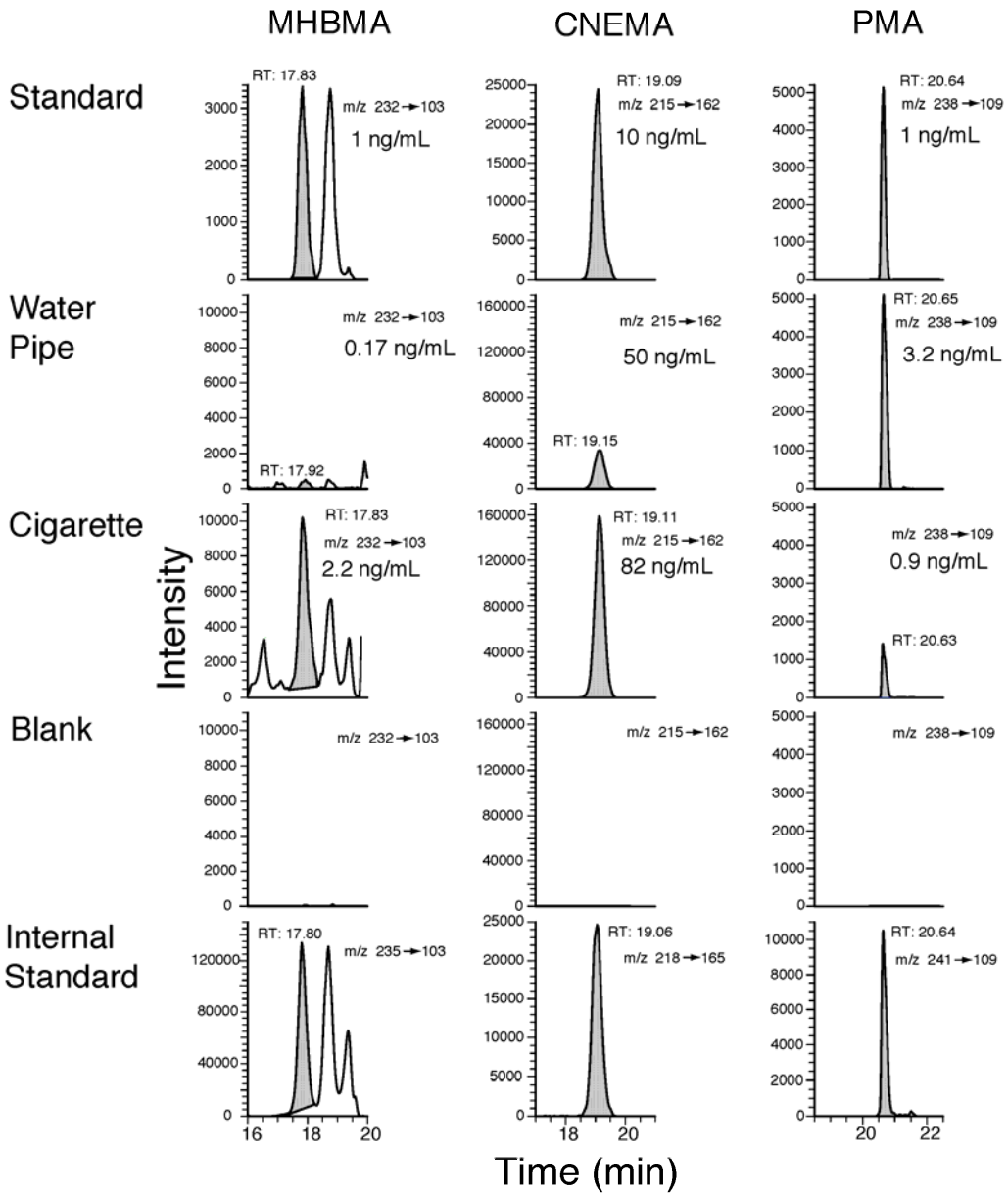

Figure S-1B. Chromatograms of urine extracts containing mercapturic acids: Metabolites of 1,3-butadiene (MHBMA), acrylonitrile (CNEMA), and benzene (PMA).

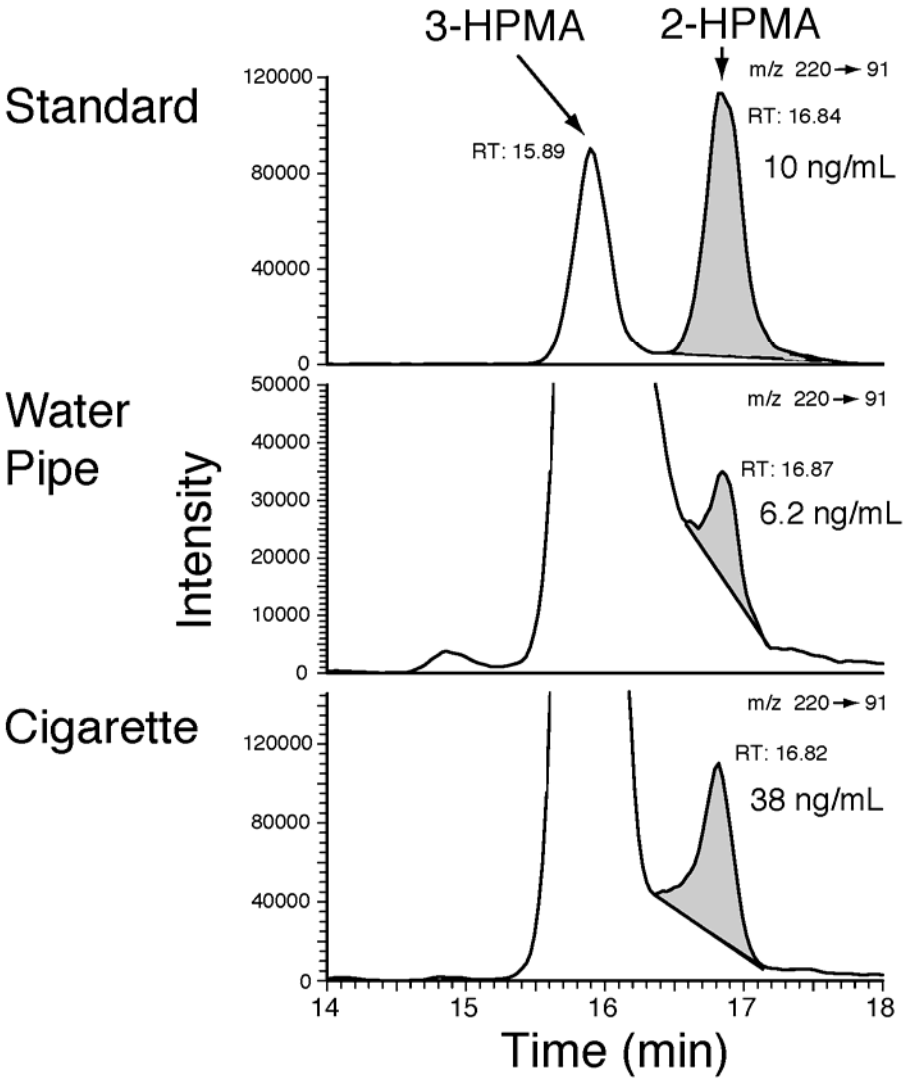

Figure S-1C. Chromatograms of urine extracts containing mercapturic acids: Metabolite of propylene oxide (2-HPMA) and separation from the isomer 3-HPMA.

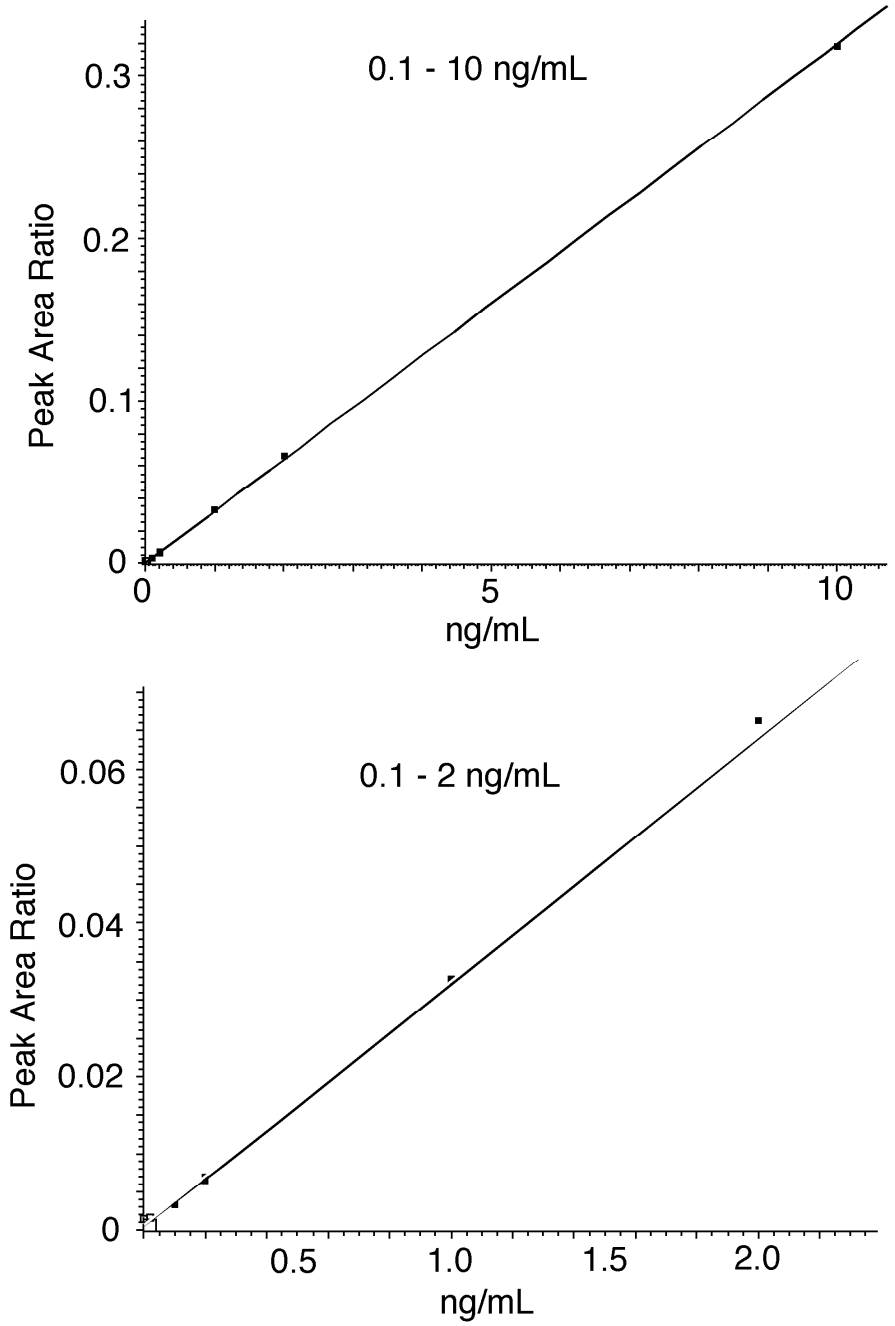

Figure S-2. Butadiene metabolite (MHBMA) calibration curves.

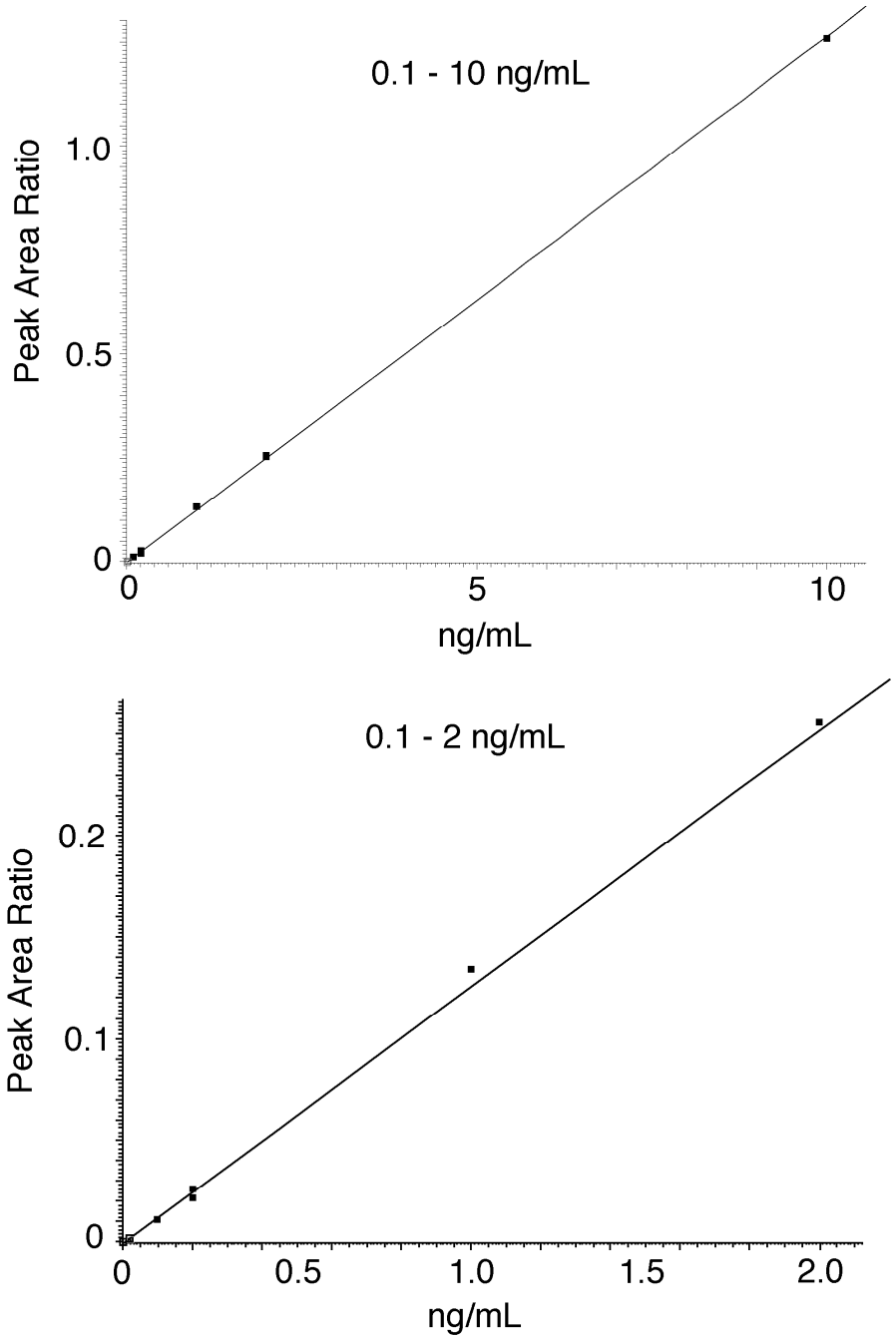

Figure S-3. Benzene metabolite (PMA) calibration curves.