Supporting Information

Identification and quantification of 1-hydroxybutene-2-yl mercapturic acid in human urine by UPLC- HILIC-MS/MS as a novel biomarker for 1,3-butadiene exposure

Katharina Sterz[1], Gerhard Scherer[1], Jan Krumsiek[2], Fabian J. Theis[2], Josef Ecker[1]*

[1]ABF Analytisch-Biologisches Forschungslabor GmbH, 80336 Munich, Germany

[2]Institute of Bioinformatics and Systems Biology, Helmholtz Zentrum Munich, 85764 Neuherberg, Germany

* Correspondence to:

Dr. Josef Ecker

ABF Analytisch-Biologisches Forschungslabor GmbH

Goethestrasse 20

80336 Munich

Germany

Tel.: +49-89-535395

Fax: +49-89-5328039

e-mail: josef.ecker@abf-lab.com

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1. Materials and Methods

Chemicals, solutions and study samples

Acetic acid (≥ 99 %), ammonium acetate (puriss. p. a.), sodium hydroxide (≥ 97%, pellets) and picric acid (1% in water) were purchased from Sigma-Aldrich (München, Germany). Methanol (optigrade), acetonitrile (optigrade) and water (optigrade) were obtained from LGC Standards (Wesel, Germany). 1-MHBMA and 2-MHBMA were obtained as mixture from Toronto Research Chemicals (North York, Canada) with purities higher than 98 % and stored at -20 °C. Also D_6-1-MHBMA and D_6-2-MHBMA were purchased as mixtures from Toronto Research Chemicals (North York, Canada) with purities higher than 98 % and stored at -20 °C. Working solutions of the desired concentrations were prepared by dilution in methanol. Urine and serum samples were obtained from a dietary controlled trial with healthy subjects, informed consent and approval from the ethics committee of the Bavarian State Board of Physicians was obtained.

Sample preparation

Aliquots of 100 µl urine were used for analysis. 10 µl of an Internal Standard solution, containing 3 ng of D_6-1-MHBMA and D_6-2-MHBMA mix were added to each sample prior to extraction. The sample (microreaction tube) was mixed and evaporated to dryness in a SpeedVac centrifuge. 100 µl of methanol were used to dissolve the residue (vortex mixer), before the sample was centrifuged for 10 min at 14,000 rpm. The supernatant was transferred to a glass vial with micro-insert.

Liquid chromatographic separation and mass spectrometric analysis

Quantitative analysis was carried out on a triple quadrupole mass spectrometer API 5000 (AB Sciex, Darmstadt, Germany), equipped with a 1200 series binary pump (G1312B), a degasser (G1379B) and a column oven (G1316B) (Agilent, Waldbronn, Germany) connected to an HTC Pal autosampler (CTC Analytics, Zwingen, Switzerland). A Turbo V ion spray source operating in negative electrospray ionization (ESI) mode was used for detection (AB Sciex, Darmstadt, Germany). High purity nitrogen was generated by a nitrogen generator NGM 22-LC/MS (cmc Instruments, Eschborn, Germany).

Chromatographic separation was performed on a Waters (Eschborn, Germany) Acquity ultra performance liquid chromatography (UPLC) BEH HILIC column (3 x 150 mm) with a 1.7 µm particle size. The column was maintained at 30°C and the injection volume was set to 2 µl. Eluent A was a 5 mM ammonium acetate buffer, eluent B consisted of acetonitrile with 5 % 100 mM ammonium acetate buffer. Gradient elution was performed with 1% A for 0.5 min, a linear increase to 7% A until 14 min, a step to 50% A until 14.01 min, hold for 2 min at 50% A until 16 min and re-equilibration from 16.01 min to 21 min with 1% A. The flow rate was set at 700 µl/min. The turbo ion spray source settings were as follows: ion spray voltage = -4.5 kV, heater temperature = 650°C, source gas 1 = 35 psi, source gas 2 = 50 psi, CAD gas = 6 psi and curtain gas = 30 psi. Analytes were monitored in the multiple reaction monitoring (MRM) mode. Quadrupoles were working at unit resolution.

Enhanced product ion spectra were generated using a Waters Acquity UPLC coupled to a Waters Xevo TQ MS tandem mass spectrometer (Waters GmbH, Eschborn, Germany). As there is only a mixture of 1- and 2- MHBMA commercially available, the isomers were chromatographically separated as described above and enhanced product ion spectra were generated at the appropriate retention times of analyte elution.

The exact mass of 1-MHBMA was analyzed in the reference substance and confirmed in real urine samples using a Waters Synapt G2-S HDMS time of flight (TOF) mass spectrometer equipped with a Acquity UPLC I-class system (Waters GmbH, Eschborn, Germany). The system was operated in the sensitivity mode (resolution: 12,000 FWHM). The analytes were separated as described above.

Calibration and Quantification

Calibration and quantification were achieved by spiking 100 µl aliquots of urine with different levels of the MHBMA standard. Calibration was performed by adding increasing amounts of the standard (Table S2) and IS as described in the sample preparation section. Calibration curves were calculated by linear regression without weighting. Data analysis was performed with Analyst software 1.5.1 (AB Sciex, Darmstadt, Germany).

Method validation

The analytical method was validated as described previously.[1] Briefly, method accuracy was calculated by using three spiked urine samples covering the entire calibration range. Precisions were determined in three un-spiked urine matrices at three different levels. For intra-day precision five samples were prepared and measured within the same day, for inter-day precisions five samples were prepared and measured on five consecutive days. Matrix effects were investigated using three individual urine samples by comparing the peak area ratios (analyte/IS) between urine samples and controls (water) at two levels.

Creatinine analysis

For urine flow normalization, creatinine was determined. 20 µl urine was diluted with 1 ml water. The sample was centrifuged at 600 rpm and the supernatant was transferred to a 96 well plate. After adding 200 µl reaction solution (0.25 M NaOH:0.1% picric acid v/v) the plate was incubated at 36°C for 45 min, subsequently absorption at 492 nm was analyzed with a Genios microplate reader (Tecan, Crailsheim, Germany). Each plate comprised a calibration set and solvent blanks.

Determination of additional biomarkers

Nicotine in urine, cotinine (main metabolite of nicotine) in serum, thiocyanate (metabolite of hydrogen cyanide) in serum, 1-OH-Pyrene (metabolite of pyrene, a polycyclic aromatic hydrocarbon/PAH) in urine, S-phenyl mercapturic acid (metabolite of benzene) in urine and 4-aminobiphenyl (a tobacco compound, an aromatic amine) were analyzed according to previously published methods. [2-6]

Data analysis and statistics

The level of significance between the groups (smokers and non-smokers) was assessed using an independent-samples Mann-Whitney U test. Biomarker data were correlated using bivariate Pearson correlations, 1-tailed. Significance of difference between the correlation coefficients was calculated using a statistical correlation difference test designed for dependent samples as described by Ramseyer et al.[7] Let r_{12} and r_{13} be the Pearson correlations of both putative biomarkers (2-MHBMA and 1-MHBMA) with a readout under investigation (e.g. nicotine), respectively. Furthermore, let r_{23} represent the pairwise correlation between the two biomarkers. Then the statistical test for the null hypothesis H_0: $r_{12}=r_{13}$ (both markers correlate equally) is given by the following z-score:

$$z = \frac{\sqrt{N\,(r_{12}-r_{13})}}{\sqrt{(1-r_{12}^2)^2+(1-r_{13}^2)^2-2r_{23}^3-(2r_{23}-r_{12}\,r_{13})(1-r_{12}^2-r_{13}^2-r_{23}^2)}}$$

where N represents the number of samples. The statistic z is approximately standard normally distributed, and the p-value can thus be obtained from the corresponding cumulative distribution function.

2. Supporting Tables

Table S1. MS parameters and RTs; (Turbo Spray IS-Voltage -4.5 kV; Source temperature 650 °C)

	MW (g/mol)	MRM (m/z)	IS MRM (m/z)	Dwell time (ms)	DP (V)	CE (V)	RT (min)
1-MHBMA	233.3	232 → 73	238 → 77	100	-40	-36	8.09
2-MHBMA	233.3	232 → 103	238 → 109	100	-40	-20	8.49

DP, declustering potential; CE, collision energy; RT, retention time.

Table S2. Calibration data

	LOD (ng/ml urine)	LOQ (ng/ml urine)	Calibration range (ng/ml urine)	IS added (ng/ml urine)	Slope	Correlation coefficient (R2)
1-MHBMA	0.05	0.15	0.12 – 7.25	1.45	0.7798	0.999
2-MHBMA	0.24	0.72	0.52 – 7.75	1.55	0.3113	0.997

Calibration lines were generated by plotting the ratios of the areas analyte/IS against the spiked concentrations (ng/ml). LOD, limit of detection; LOQ, limit of quantification.

Table S3. Accuracies, intra-day and inter-day precisions

	Spiked (ng/ml urine)	Accuracy (%)	Concentration (ng/ml urine)	Intra-day (n = 5)		Inter-day (n = 6)	
				(ng/ml ± SD)	CV (%)	(ng/ml ± SD)	CV (%)
1-MHBMA	0.24	102.4	0.24	0.25± 0.02	6.10	0.25 ± 0.02	7.32
	1.21	102.9	1.21	1.29 ± 0.04	3.23	1.27 ± 0.04	3.14
	4.83	98.0	4.83	4.90 ± 0.13	2.67	4.86 ± 0.14	2.80
2-MHBMA	0.52	93.0	0.52	0.46 ±0.04	7.36	0.50 ± 0.10	19.68
	1.29	107.0	1.29	1.34 ± 0.13	9.42	1.42 ± 0.14	10.13
	5.17	85.3	5.17	4.27 ± 0.17	3.94	4.46 ± 0.19	4.37

The displayed accuracy is the mean of the assayed concentration (corrected by endogenous levels of the urine samples) in percent of the actual spiked concentration. For intra-day precision the mean concentrations and CVs of five individual samples, for inter-day precision mean concentrations and CVs of six individual samples is shown. For recovery the mean percent from three individual extractions of recovered compound compared to spiked controls is shown.

Table S4. Assessment of matrix effects

	1-MHBMA		*2-MHBMA*	
	Analyte/ IS	% Diff. from cont.	Analyte/IS	% Diff. from cont.
Low	0.24 ng/ml		0.52 ng/ml	
Control	0.132	-	0.16	-
Urine A	0.126	-4.9	0.18	12.4
Urine B	0.150	13.4	0.14	-11.0
Urine C	0.136	2.6	0.14	-11.8
High	4.83 ng/ml		5.17 ng/ml	
Control	3.59	-	2.93	-
Urine A	3.70	3.1	2.62	-10.5
Urine B	3.37	-5.9	3.12	6.4
Urine C	3.53	-1.6	2.85	-2.8

Matrix effects were investigated using three individual urine samples by comparing the peak area ratios between urine samples and controls (water) at two levels. Displayed are the peak area ratios of spiked standards at two levels in urine samples and control and the percent difference compared to control. The percentage of area ratios difference for three different urine matrices compared to non-matrix samples ranged from -10.5 % to 13.4 % demonstrating that no significant matrix effect is present. IS, internal standard; diff; difference; cont., control.

Table S5. Sample stabilities

	Concentration (ng/ml urine)	Stability RT; conc. (%) after 24 h	F/T stability; conc. (%) after 6 cycles	Stability of prepared sample at 10°C; conc. (%) after 3 d
1-MHBMA	0.20	94.8	93.0	94.5
	5.00	111.9	92.1	95.8
2-MHBMA	0.57	100.7	95.2	96.3
	4.30	96.7	111.7	91.2

Numbers represent percent concentrations after the indicated conditions and periods. RT, room temperature; conc., concentration.

3. Supporting Figures

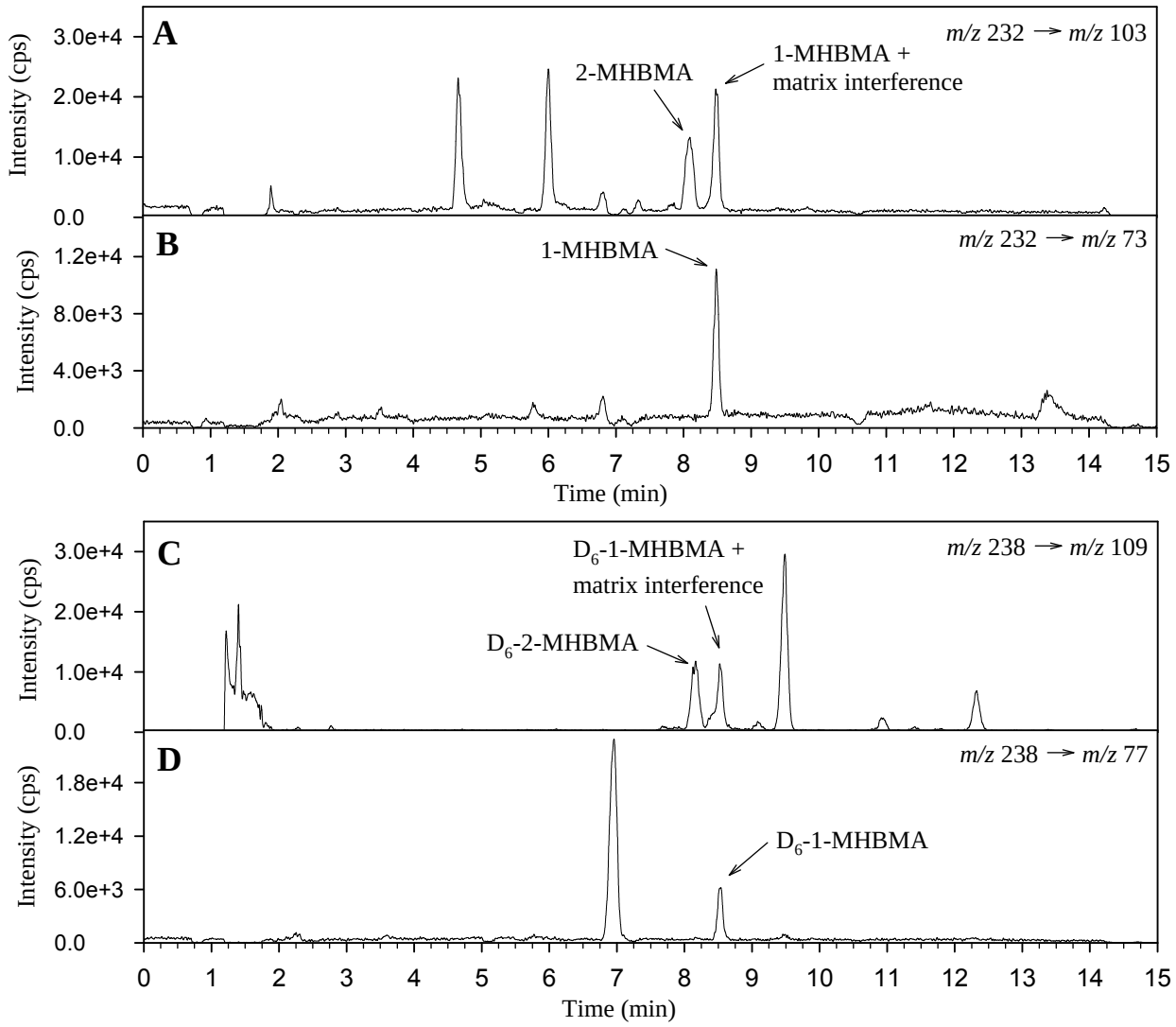

Figure S1. MRM chromatograms of a urine sample spiked with 1.99ng/ml 1-MHBMA and 1.91 ng/ml 2-MHBMA. (A) 2-MHBMA: MRM 232/103; (B) 1-MHBMA: MRM 232/73; (C) D6-2-MHBMA: MRM 238/109; (D) D6-1-MHBMA: 238/77.

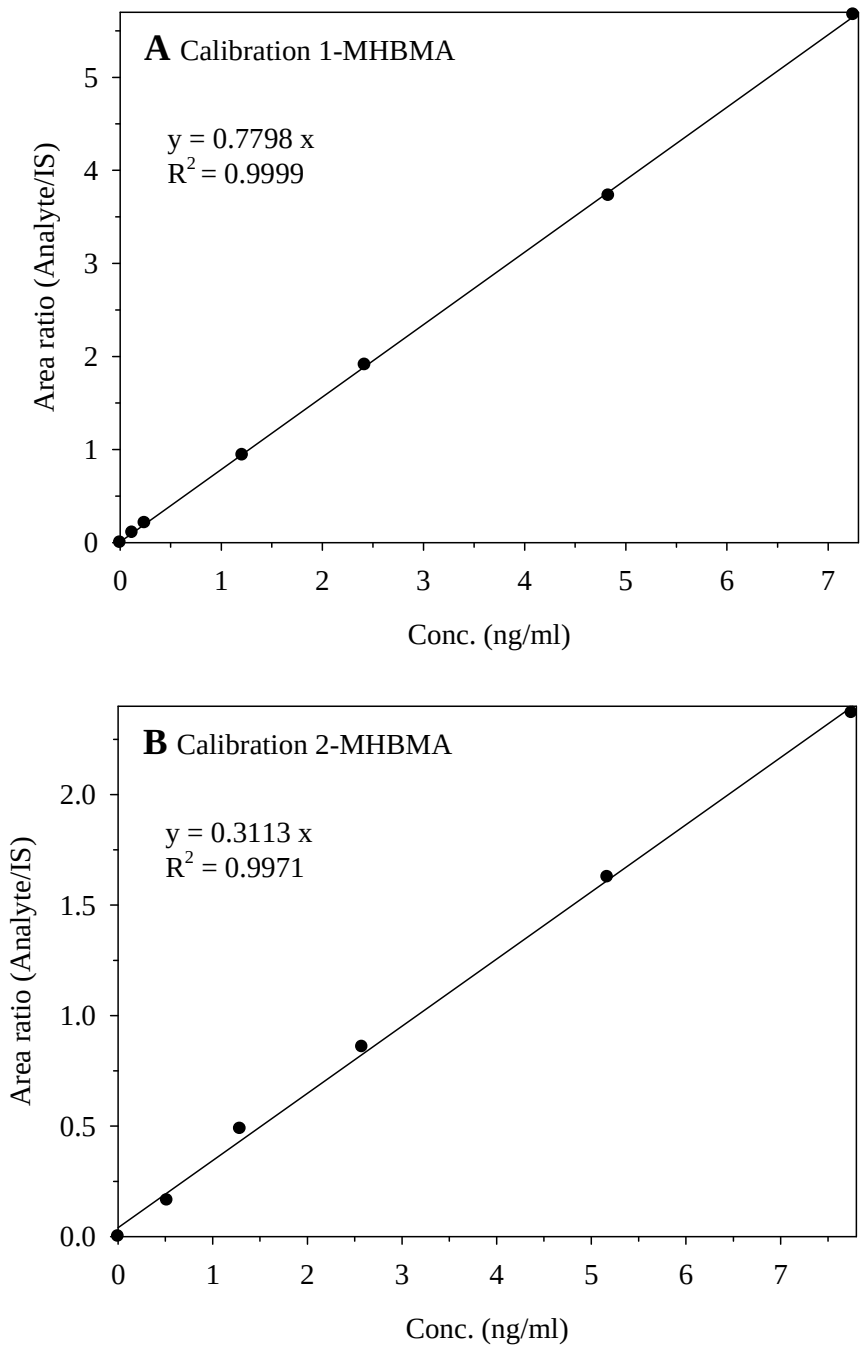

Figure S2. Calibration curves for (A) 1-MHBMA and (B) 2-MHBMA.

4. Supporting References

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