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# Rapid and Sensitive HILIC-ESI-MS/MS Quantitation of Polar Metabolites of Acrylamide in Human Urine Using Column Switching with an Online Trap Column

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The carcinogen acrylamide (AA) is formed during the processing of food. AA is metabolized to mercapturic acids, which are excreted with urine. A hydrophilic interaction liquid chromatography tandem mass spectrometry method (HILIC-MS/MS) using a zwitterionic stationary phase (Zic-HILIC) was developed and validated to quantitate the mercapturic acids of AA (AAMA) and glycidamide (GAMA), and AAMA-sulfoxide in human urine. In contrast to reversed phases, the application of Zic-HILIC resulted in efficient retention and separation of these highly polar compounds. Off-line sample workup was avoided by application of column switching with a Stability BS-C17 trap column prior to the analytical column, thus minimizing interferences with the urinary matrix. Limit of quantification values (LOQs) were 0.5 $\mu$g/L (AAMA), 2.0 $\mu$g/L (AAMA-sulfoxide), and 1.0 $\mu$g/L (GAMA) in human urine. Median concentrations in urine samples ($n = 54$) of six nonsmoking human subjects were 24.0 $\mu$g/L (AAMA, 7.8−79.8 $\mu$g/L), 16.7 $\mu$g/L (AAMA-sulfoxide, 6.8−70.1 $\mu$g/L), and 3.82 $\mu$g/L (GAMA, 1.0−23.6 $\mu$g/L).

**KEYWORDS: Acrylamide; mercapturic acids; sulfoxides; HILIC; mass spectrometry; human urine**

## INTRODUCTION

Acrylamide (AA, **Figure 1**) is a carcinogen in rodents (*1*−*3*) and is classified as a probable human carcinogen (*4*). AA is generated in concentrations up to several ppm during the heat treatment of carbohydrate-rich foods (*5*−*9*), and concern has been raised regarding health risks of AA-exposure with food.

On the basis of food consumption patterns and AA-concentrations in food, the average daily AA-intake in the US is estimated as approximately 0.4 $\mu$g AA/kg b.w. with a 90th percentile of 0.95 $\mu$g/kg b.w. (*10*). In children and adolescents, the daily intake may be 1.5 times higher because of differing food consumption patterns (*11*). Such indirect exposure assessments may be supported by biomonitoring, which is considered more precise because of a direct determination of the analyte of interest or relevant biotransformation product in human subjects (*12, 13*). However, for correct estimates, a detailed knowledge of the toxicokinetics of the compounds is required (*14*).

Toxicokinetics also have a major influence on the toxicity of AA. After oral administration to rodents, AA is readily absorbed and distributed (*15, 16*), and 65% of the administered AA-dose is conjugated with glutathione (*17*). After metabolic processing, the formed cysteine *S*-conjugate, *N*-acetyl-*S*-(2-carbamoylethyl)-L-cysteine (AAMA) is excreted in urine. In part, AA is oxidized to glycidamide (GA) (*18*−*20*), which also reacts with glutathione. rac-*N*-Acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA) and rac-*N*-acetyl-*S*-(1-carbamoyl-2-hydroxyethyl)-L-cysteine (iso-GAMA) are excreted as urinary metabolites that result from the oxidative pathway. In humans, AAMA is also sulfoxidized to AAMA-sulfoxide (*21*) (**Figure 1**).

Because of high sensitivity and specificity, LC-MS/MS is the method of choice for analysis of metabolites present in low concentrations in biological fluids. However, many metabolites excreted in urine such as the highly polar AA-metabolites are not well retained on reversed phase (RP) materials and usually elute close to or with the void volume. Moreover, due to the presence of salts and other polar components in urine, retention time shifts and ion suppression result in major difficulties in quantitation of such polar analytes in urine.

Several methods for the quantification of AAMA and GAMA in urine using RP columns therefore apply a time-consuming sample workup (*22*−*25*). Quantification of AAMA and GAMA by LC-MS/MS using column switching has been reported (*26*). The aim of this study was to establish a reliable, highly sensitive, and specific analytical method to simultaneously quantify polar analytes using the AA-metabolites AAMA, AAMA-sulfoxide, and GAMA as relevant examples. By monitoring these metabolite levels in urine samples of humans unintentionally exposed to AA from the diet, conclusions on the daily dietary intake of acrylamide may be made.

## MATERIALS AND METHODS

**Chemicals.** Water and acetonitrile were purchased from Roth (Karlsruhe, Germany). $^{13}C_3$-acrylamide (chemical purity >98%, isotope

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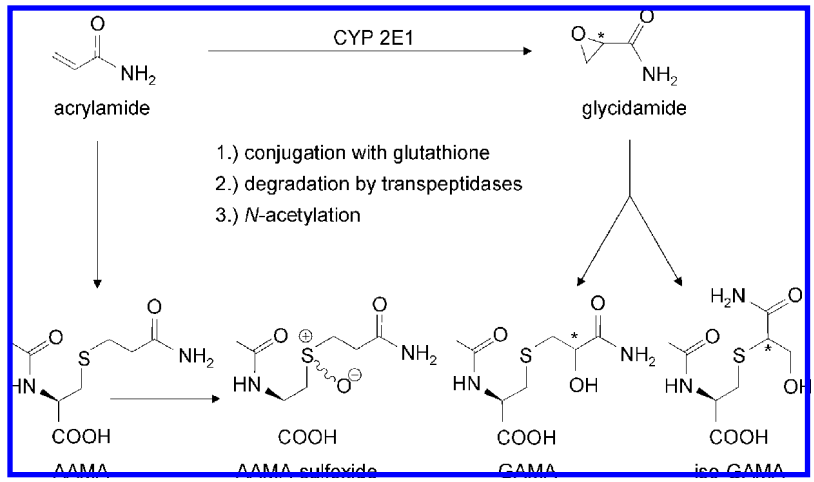


**Figure 1.** Biotransformation of acrylamide in humans.

enrichment >99%) was purchased from CK Gas Products Ltd. (Hook, Hampshire, UK). Glycidamide (chemical purity >98%) was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). All other chemicals were from Sigma/Fluka (Taufkirchen, Germany) in the highest purity available, and all solvents used were HPLC grade.

**Syntheses.** $^{13}C_3$-Glycidamide was obtained by oxidation of $^{13}C_3$-acrylamide with dimethyldioxirane (*27*) in acetone (*28*). Synthesis of the mercapturic acids of AA, $^{13}C_3$-AA, GA, and $^{13}C_3$-GA was performed in two steps. In the first step, the cysteine conjugates *S*-(2-carbamoylethyl)-L-cysteine (AA-Cys) and $^{13}C_3$-*S*-(2-carbamoylethyl)-L-cysteine ($^{13}C_3$-AA-Cys), and the regioisomers *S*-(1-carbamoyl-2-hydroxyethyl)-L-cysteine and *S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (summarized as GA-Cys) as well as $^{13}C_3$-*S*-(1-carbamoyl-2-hydroxyethyl)-L-cysteine and $^{13}C_3$-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (summarized as $^{13}C_3$-GA-Cys) were synthesized (*29*). In the second step, AA-Cys, $^{13}C_3$-AA-Cys, GA-Cys, and $^{13}C_3$-GA-Cys (both regioisomers) were acetylated with acetic anhydride (*26*) using acetic acid as solvent. $^2H_3$-AAMA and $^2H_3$-GAMA were synthesized analogous to AAMA and GAMA using $^2H_6$-acetic anhydride and $^2H_3$-acetic acid. AAMA, $^{13}C_3$-AAMA, and $^2H_3$-AAMA were transformed to sulfoxides by oxidation with $H_2O_2$ (*30*).

All synthesized chemicals were purified by HPLC (Reprosil-Pur C18 Aq, 5 $\mu$m, 150 mm × 4.6 mm, Dr. Maisch, Ammerbuch, Germany). The compounds were dissolved in water at concentrations of approximately 1 mg/mL, and samples of 50−100 $\mu$L were injected in a series of separations. One-sixth of the total flow of 0.75 mL/min (isocratic elution with a mixture of 98% of water containing 0.1% formic acid and 2% of acetonitrile) was split into the mass spectrometer. By monitoring the specific mass transitions (**Table 1**) for the compounds to be isolated, fractions were collected. An electrical valve (Valco Valve) was applied to automatize the procedure.

Identity of the synthetic products was confirmed by comparison of their mass spectra to those previously reported (*21, 26*). $^2H_3$-AAMA-sulfoxide was characterized by mass spectrometry in the negative ion mode after direct infusion. The precursor ion *m/z* 252.1 [M − H$^-$] fragmented to *m/z* 180.9, 120.0, 119.0, 75.0, 59.1, and 49.0. The purity of all synthesized compounds was determined to be >95% by $^1$H NMR as described previously (*21, 26, 31*). In comparison to unlabeled AAMA-sulfoxide, the $^1$H NMR of $^2H_3$-AAMA-sulfoxide did not show a signal for the L-cysteine methyl group at $\delta$ = 2.00 ppm (3H, s, $CH_3$) because of the exchange of $^1$H with $^2$H.

**Urine Samples.** Urine samples were obtained from healthy human subjects (three female and three male subjects, body weights between 52 and 75 kg, and age between 23 and 28 years). Urine samples from the subjects were collected over a predetermined time frame of 72 h in intervals of 8 h (0−8, 8−16, 16−24, 24−32, 32−40, 40−48, 48−56, 56−64, and 64−72 h). All subjects were nonsmokers and did not drink alcoholic beverages 72 h before and during the study. The participants of the study were asked to avoid known major dietary AA sources such as potato chips and French fries while otherwise keeping to their usual dietary habits. Individual urine volumes were recorded, and five aliquots were stored at −20 °C until analysis. In accordance with the Declaration of Helsinki, the study was performed with informed consent, and the study protocol was reviewed and approved by the local institutional review board.

**Table 1.** MS/MS-Transitions, Declustering Potentials (DP), Collision Energies (CE), and Cell Exit Potentials (CXP) Used for the Detection of AAMA, GAMA, and AAMA-Sulfoxide in Human Urine

| transition [M − H]$^-$ | compound | DP (V) | CE (V) | CXP (V) |
|---|---|---|---|---|
| 233.2 → 104.0 | AAMA (quantifier) | −26 | −24 | −5 |
| 233.2 → 58.0 | AAMA (qualifier) | −26 | −52 | −7 |
| 236.2 → 107.0 | $^{13}C_3$-AAMA (calibration curve) | −21 | −18 | −15 |
| 236.2 → 104.0 | $^2H_3$- AAMA (internal standard) | −16 | −22 | −5 |
| 249.2 → 120.0 | GAMA[a] (quantifier) | −26 | −22 | −7 |
| 249.2 → 128.0 | GAMA (qualifier) | −26 | −18 | −7 |
| 249.2 → 75.0 | GAMA (qualifier) | −26 | −48 | −1 |
| 249.2 → 73.0 | iso-GAMA (qualifier) | −26 | −48 | −11 |
| 252.2 → 123.0 | $^{13}C_3$[b]-GAMA (calibration curve) | −21 | −24 | −11 |
| 252.2 → 120.0 | $^2H_3$[c]-GAMA (internal standard) | −21 | −24 | −9 |
| 249.2 → 116.0 | AAMA-sulfoxide (quantifier) | −16 | −22 | −9 |
| 249.2 → 178.0 | AAMA-sulfoxide (qualifier) | −16 | −14 | −9 |
| 252.2 → 116.0 | $^{13}C_3$-AAMA-sulfoxide (calibration curve) | −21 | −24 | −9 |
| 252.2 → 119.0 | $^2H_3$-AAMA-sulfoxide (internal standard) | −21 | −22 | −9 |

[a] This transition accounts for GAMA and iso-GAMA as well. Both metabolites are quantitated as Σ of GAMA. [b] This transition accounts for both $^{13}C_3$-GAMA and $^{13}C_3$-iso-GAMA. [c] This transition accounts for both $^2H_3$-GAMA and $^2H_3$-iso-GAMA.

**Sample Preparation.** Frozen samples were thawed, vortexed, and diluted with an equal volume of acetonitrile. To remove precipitated proteins, samples were centrifuged for 10 min at 4 °C and 1,400*g* followed by the addition of the internal standards (IS) at a final concentration of 30 $\mu$g/L of each analyte. The IS-solution was prepared by dissolving $^2H_3$-AAMA, $^2H_3$-GAMA, and $^2H_3$-AAMA-sulfoxide in water at a concentration of 3,000 $\mu$g/L.

**Hydrophilic Interaction Liquid Chromatography (HILIC).** The Stability BS-C17 trap column (5 $\mu$m, 3 mm × 33 mm, Dr. Maisch, Ammerbuch, Germany) was preconditioned for 60 min with a mixture of 80% ACN and 20% ammonium acetate buffer (20 mM, pH 6.9) at a flow rate of 1.0 mL/min and subsequently equilibrated to 90% ACN and 10% ammonium acetate buffer for 45 min. For online cleanup of urine samples prior to HILIC analysis, an electrical valve (Valco Valve) was applied to control the flow of the solvents from two pumps into two different columns. In the first step, the autosampler (Agilent Series 1100, Waldbronn, Germany) introduced the sample (100 $\mu$L) into the system, and pump 1 (Agilent Series 1100, Waldbronn, Germany) delivered the mobile phase, consisting of 10% ammonium acetate buffer (20 mM, pH 6.9) and 90% acetonitrile at a flow rate of 1.0 mL/min to

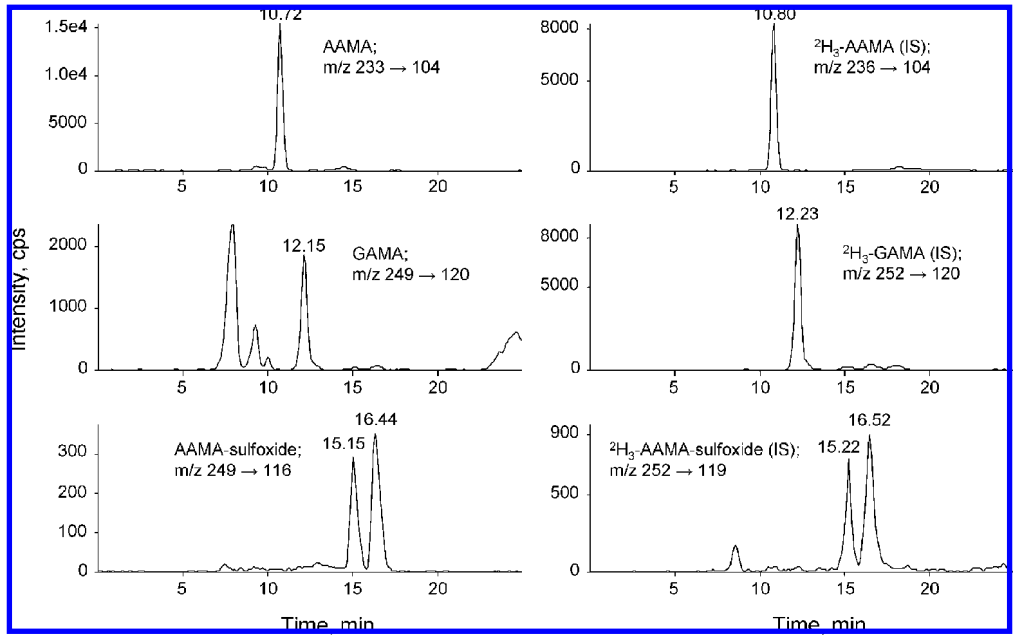


**Figure 2.** Chromatogram showing the mass traces for the mercapturic acids of acrylamide excreted in the urine of a nonintentionally exposed human subject. The corresponding mass traces for the internal standards are shown on the right-hand side. Analyzed by HILIC-ESI$^-$-MS/MS in the multiple reaction monitoring mode (MRM).

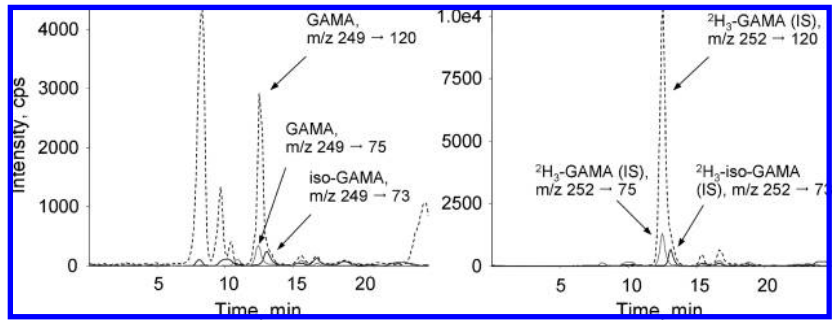


**Figure 3.** Chromatogram showing the mass traces for the regioisomers GAMA (gray line) and iso-GAMA (black line) as well as the mass trace accounting for both metabolites (dotted line) in the urine of a nonintentionally exposed human subject. By monitoring the common mass transition *m/z* 249 → 120, both regioisomers could be conjointly quantitated. The mass traces for the corresponding internal standards are shown on the right-hand side.

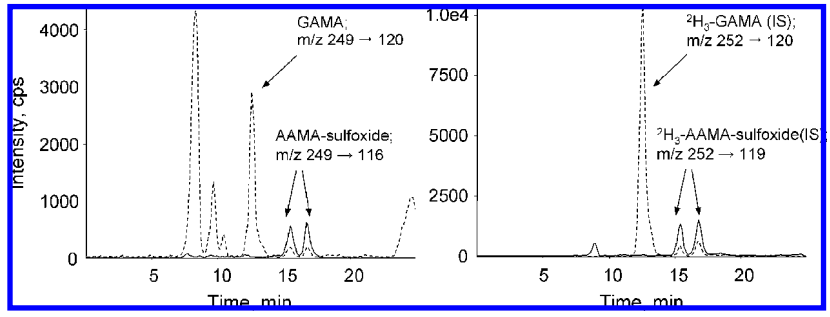


**Figure 4.** Chromatographic baseline separation of the isobaric urinary metabolites GAMA (dotted line) and AAMA-sulfoxide (black line). The mass traces for the corresponding internal standards are shown on the right-hand side.

load the sample onto the trap column. Sample loading and elimination of matrix components were completed after 3 min. Then, the valve switched to the eluting position, and pump 2 (Agilent Series 1100) flushed the trapped analytes at 0.4 mL/min in the reverse direction from the trap column to the analytical column (Zic-HILIC, 3.5 $\mu$m, 2.1 mm $\times$ 150 mm, SeQuant AB, Umeå, Sweden). The applied solvent mixture consisted of 14% ammonium acetate buffer and 86% acetonitrile. After 6 min, the valve was switched back to loading position to prevent the transfer of trapped matrix components to the analytical column. The trap column was flushed with 10% ammonium acetate buffer and 90% acetonitrile for another 10 min, and analysis was completed after 25 min.

**Mass Spectrometry.** A triple-stage quadrupole mass spectrometer (API 3000, Applied Biosystems, Darmstadt, Germany) equipped with an electrospray ionization source was used. Ion spray voltage was $-3{,}100$ V with a source temperature of 400 °C using nitrogen as curtain and collision gas. Focusing potential was set to $-200$ V and entrance potential to $-10$ V. Negative ions were analyzed by multiple reaction monitoring (MRM) with a dwell time of 100 ms for each transition. Analyte specific acquisition parameters were obtained by infusion of standards using the quantitative optimization function of the Analyst 1.4.1 software (Applied Biosystems) (**Table 1**). At least two transitions were monitored for each analyte and MRMs with the highest relative response, and specificities were used for quantification.

**Table 2.** Analytical Performance for the Determination of $^{13}C_3$-AAMA, $^{13}C_3$-GAMA, and $^{13}C_3$-AAMA-Sulfoxide in Human Urine[a]

| | $^{13}C_3$-AAMA | | | | $^{13}C_3$-GAMA | | | | $^{13}C_3$-AAMA-sulfoxide | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| expected concentration ($\mu$g/L) | mean ($\mu$g/L) | S.D. | %CV | Accy | mean ($\mu$g/L) | S.D. | %CV | Accy | mean ($\mu$g/L) | S.D. | %CV | Accy |
| intraday | | | | | | | | | | | | |
| 5 | 4.82 | 0.36 | 7.4 | 96.5 | 4.70 | 0.33 | 7.0 | 94.1 | 4.75 | 0.16 | 3.4 | 95.1 |
| 50 | 49.97 | 1.79 | 3.6 | 99.9 | 52.40 | 4.09 | 7.8 | 104.8 | 50.10 | 0.10 | 0.2 | 100.2 |
| 500 | 519.67 | 20.13 | 3.9 | 103.9 | 544.67 | 37.00 | 6.8 | 108.9 | 575.00 | 10.44 | 1.8 | 115.0 |
| interday | | | | | | | | | | | | |
| 5 | 4.85 | 0.31 | 6.4 | 97.0 | 4.58 | 0.44 | 9.6 | 91.5 | 4.29 | 0.65 | 15.2 | 85.8 |
| 50 | 49.67 | 2.29 | 4.6 | 99.3 | 52.90 | 3.50 | 6.6 | 105.8 | 48.50 | 2.69 | 5.5 | 97.0 |
| 500 | 528.33 | 23.69 | 4.5 | 105.7 | 561.33 | 8.33 | 1.5 | 112.3 | 548.00 | 53.51 | 9.8 | 109.6 |

[a] Mean and standard deviation (S.D.; each standard sample for the calibation was analyzed three times), interday and intraday precision (coefficient of variation, CV) and accuracy (Accy).

**Table 3.** Precision Data for the Determination of AAMA, GAMA, and AAMA-Sulfoxide in 50 Individually Prepared Quality Control Samples of the Same Urine (Test of Robustness)[a]

| | | AAMA | GAMA | AAMA-sulfoxide |
|---|---|---|---|---|
| quality control samples ($n = 50$) | mean ($\mu$g/L) | 14.42 | 2.56 | 12.41 |
| | S.D. | 0.91 | 0.30 | 1.63 |
| | %CV | 6.31 | 11.83 | 13.09 |

[a] Mean and standard deviation (S.D.; $n = 50$) and precision (coefficient of variation, CV).

**Calibration Curves and Quantification.** Calibration samples were prepared in human urine as matrix with concentrations of $^{13}C_3$-AAMA, $^{13}C_3$-GAMA, and $^{13}C_3$-AAMA-sulfoxide ranging from 0.5 to 1 000 $\mu$g/L. Calibration curves were calculated by linear regression and weighted $1/x$ using the Analyst 1.4.2 software (Applied Biosystems). Because of an identical response for $^{13}$C-labeled and $^{12}$C-analytes, AAMA, GAMA, and AAMA-sulfoxide contents in urine were calculated from the linear regression coefficients obtained with the $^{13}$C-labeled compounds.

**Method Validation.** The method was characterized for dynamic range, limit of detection (LOD, defined by a signal-to-noise ratio $S/N \geq 3$), and limit of quantification (LOQ, $S/N \geq 10$). LODs and LOQs were determined by analysis of urine by adding increasing concentrations of $^{13}C_3$-AAMA, $^{13}C_3$-GAMA, and $^{13}C_3$-AAMA-sulfoxide. For intraday and interday precision and accuracy, urine samples (26.5 mg/dL creatinine) were spiked with three different concentrations (5 $\mu$g/L, 50 $\mu$g/L, and 500 $\mu$g/L) of $^{13}C_3$-labeled analytes, split into aliquots and stored at −20 °C. To assess intraday precision and accuracy, the three concentrations were prepared, and all samples were measured in triplicate. For interday precision and accuracy, determinations of the three concentrations were performed on three different days with new samples prepared each day from the frozen aliquots. Additionally, quality control samples ($n = 50$) were analyzed continuously along with the analyses. Each quality control sample was prepared individually from the same urine sample. The samples were analyzed for AAMA, GAMA, and AAMA-sulfoxide on 9 different days, thus providing further data on long-term stability of the analytical method.

**Creatinine Analysis.** All urine samples were analyzed for content of creatinine. Quantitation of creatinine was performed with a Cobas Integra system creatinine plus ver. 2 (Roche Diagnostics, Mannheim, Germany).

## RESULTS AND DISCUSSION

**Method Development.** Because of poor retention on traditional RP phases for HPLC and ion suppression by coeluting matrix components, quantification of hydrophilic compounds such as the urinary metabolites of AA by LC-MS/MS is very difficult. In HILIC, highly polar analytes are retained on the column by partitioning between a water-enriched layer attracted by the polar stationary phase and the solvent, consisting of a mixture of acetonitrile and aqueous buffer (e.g., ammonium acetate) in the range of 5−40%. The retention capabilities of HILIC material increase with hydrophilicity and polarity of the analyte. Also, an increasing concentration of organic solvent in the mobile phase increases retention (*32, 33*). The zwitterionic sulfoalkylbetaine stationary phase Zic-HILIC additionally offers the possibility of a more selective separation of charged analytes by weak electrostatic interactions with the column surface.

Because of the ability to separate polar analytes, HILIC technology has recently been used for metabonomic studies in human and rat urine (*34, 35*). Quantitation of single analytes in urine with HILIC-MS/MS, however, still requires sample preparation procedures such as SPE because of interferences of polar matrix components (*36, 37*). To minimize the influence of the urinary matrix and to increase the on-column concentration of the analytes, a simple online cleanup procedure was developed. By trapping the analytes on a Stability BS-C17 trap column and reversing the solvent flow, most of the interfering matrix components were removed. Although the BS-C17 material, a mixed mode RP/SAX (strong anion exchange) phase, was not specifically designed for HILIC, retention time also increased with decreasing water content in the mobile phase, indicating a HILIC-like mode of retention.

By applying this trap column in combination with a Zic-HILIC analytical column and MS/MS, a rapid, reliable, sensitive, and specific analytical method for the simultaneous quantification of all relevant urinary metabolites of AA was possible. AAMA, AAMA-sulfoxide, and GAMA showed excellent retention on the Zic-HILIC column (**Figure 2**) and were well separated, including the regioisomers GAMA and iso-GAMA (**Figure 3**) and the two diastereomers of AAMA-sulfoxide (**Figure 4**). The analytical column showed excellent performance over more than 1,000 analytical runs. Deterioration of the column material was first indicated by deformation of peak shapes. The trap column was changed every 400 runs.

Because of the baseline separation of the isobaric AAMA-sulfoxide and GAMA, which share a fragmentation of $m/z$ 249.2 to $m/z$ 120.0, interferences between these metabolites could be avoided. Peaks representing AAMA-sulfoxide may easily be misinterpreted as GAMA if the peaks are not clearly separated. An inefficient separation of GAMA and AAMA-sulfoxide may explain some of the high GAMA concentrations reported in human urine samples (*24, 31*). GA, the precursor of GAMA, is the DNA-reactive AA-metabolite supposedly responsible for tumor induction after AA administration in rodents (*38*−*40*). Therefore, an overestimation of GAMA excretion due to interference with AAMA-sulfoxide in human urine may result in to an overestimation of the potential risk of health effects due to AA exposures for humans.

**Method Performance.** Retention times in HILIC measurements vary strongly depending on the matrix, and retention time shifts of up to several minutes were observed when analyzing

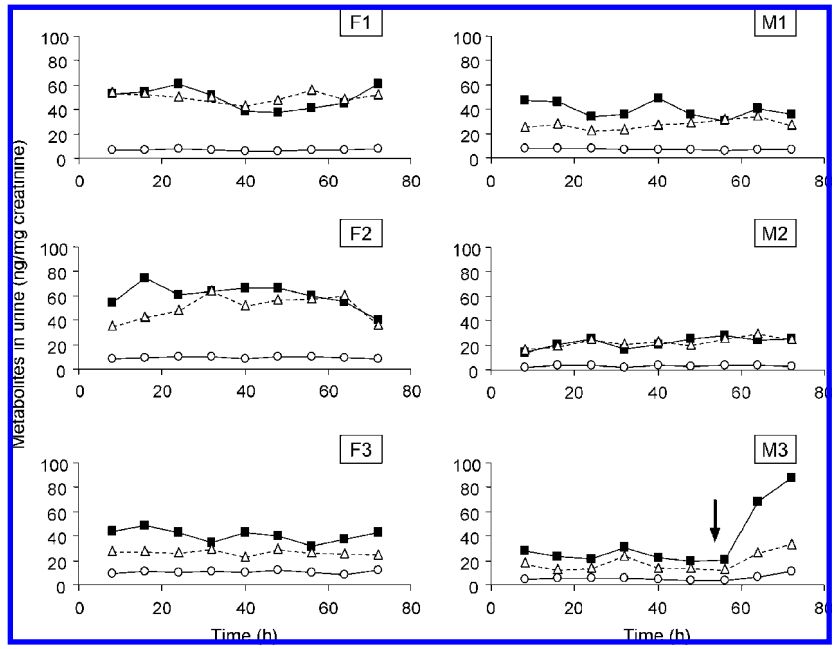


**Figure 5.** Urinary excretion of AAMA (■), AAMA-sulfoxide (Δ), and GAMA (○) monitored in the urines of female (F1−F3) and male (M1-M3) human subjects over 9 consecutive sampling intervals. The arrow indicates the ingestion of French fries by participant M3.

**Table 4.** Concentrations of AAMA, AAMA-Sulfoxide, and GAMA of Human Subjects Because of the Presence of Acrylamide in Food

| participant | AAMA (μg/L) | AAMA-sulfoxide (μg/L) | GAMA (μg/L) |
|---|---|---|---|
| F1 ($n = 9$) | | | |
| median | 22 (52)[a] | 27 (50)[a] | 4 (6)[a] |
| range | 10 − 72 (37 − 61)[a] | 11 − 70 (43 − 56)[a] | 1 − 9 (6 − 8)[a] |
| F2 ($n = 9$) | | | |
| median | 36 (61)[a] | 31 (52)[a] | 7 (10)[a] |
| range | 11 − 64 (40 − 74)[a] | 10 − 55 (35 − 64)[a] | 2 − 11 (8 − 11)[a] |
| F3 ($n = 9$) | | | |
| median | 37 (43)[a] | 23 (27)[a] | 9 (10)[a] |
| range | 15 − 78 (31 − 48)[a] | 11 − 57 (24 − 29)[a] | 4 − 24 (9 − 12)[a] |
| M1 ($n = 9$) | | | |
| median | 25 (36)[a] | 15 (28)[a] | 4 (7)[a] |
| range | 8 − 47 (30 − 49)[a] | 10 − 32 (23 − 35)[a] | 2 − 11 (5 − 8)[a] |
| M2 ($n = 9$) | | | |
| median | 16 (25)[a] | 14 (23)[a] | 2 (3)[a] |
| range | 8 − 38 (14 − 29)[a] | 8 − 37 (16 − 30)[a] | 1 − 7 (2 − 4)[a] |
| M3 ($n = 9$) | | | |
| median | 14 (24)[a] | 10 (14)[a] | 3 (5)[a] |
| range | 11 − 80 (20 − 88)[a] | 7 - 31 (13 - 34)[a] | 2 − 10 (4 − 11)[a] |
| overall ($n = 54$)[b] | | | |
| median | 24 (40)[a] | 17 (28)[a] | 4 (7)[a] |
| range | 8 − 80 (14 − 88)[a] | 7 − 70 (13 − 64)[a] | 1 − 24 (2 − 12)[a] |

[a] Urinary concentrations related to creatinine (ng/mg creatinine). [b] Median and range calculated from all 54 samples derived from the six individuals over 9 consecutive sampling periods.

standards of AA metabolites using pure water or human urine samples as matrix. Therefore, the column switching method was not applicable to generate calibration curves for the AA metabolites in water, and LODs and LOQs needed to be determined in matrix (human urine samples). Using the column switching method, retention time shifts between different human urine samples were negligible. Since all human urine samples contain AA-metabolites in varying concentrations, calibration curves were generated using $^{13}C_3$-labeled analytes, thus avoiding extrapolation from background levels or complicated standard addition procedures. The obtained linear regression coefficients were used for the calculation of metabolite concentrations since identical concentrations of $^{13}C_3$-labeled and unlabeled standards gave identical peak areas (data not shown). The slopes of the calibration curves were 0.022 ($^{13}C_3$-AAMA), 0.0135 ($^{13}C_3$-GAMA), and 0.00582 ($^{13}C_3$-AAMA-sulfoxide). Calibration curves for $^{13}C_3$-AAMA, $^{13}C_3$-GAMA, and $^{13}C_3$-AAMA-sulfoxide were linear up to concentrations of 1,000 μg/L with correlation coefficients $r^2 = 0.9998$, 0.9998, and 0.9995, respectively. LODs were 0.1 μg/L ($^{13}C_3$-AAMA), 0.5 μg/L ($^{13}C_3$-GAMA), and 1 μg/L ($^{13}C_3$-AAMA-sulfoxide), and LOQs were 0.5 μg/L ($^{13}C_3$-AAMA), 1 μg/L ($^{13}C_3$-GAMA), and 2.0 μg/L $^{13}C_3$-AAMA-sulfoxide. The method also showed high interday and intraday precision, accuracy, and reproducibility (**Tables 2** and **3**).

**Concentrations of AA-Metabolites in Human Urine Samples.** For biomonitoring of average AA-metabolite excretion, urine from six human subjects was collected over 72 h in 8 h intervals (**Figure 5**). The median values of AA-metabolites excreted with urine (μg/L and ng/mg creatinine) give a good estimate of the average exposures of the human subjects to AA (**Table 4**) since variations in urinary concentrations of AA-

**Table 5.** Comparison of Reported Acrylamide Metabolite Excretion with Human Urine Because of Acrylamide Exposure from the Diet and/or Cigarette Smoke

| exposure | AAMA[a] | AAMA-sulfoxide[h] | GAMA | GA:AA[b] | excretion of AA-equivalents(μg /24 h)[c] | reference |
|---|---|---|---|---|---|---|
| nonsmokers ($n = 16$) | 29 μg/L | ND | 5 μg/L[d] | 0.22 | NC | ref *23* |
| smokers ($n = 13$) | 127 μg/L | ND | 19 μg/L | 0.15 | NC | |
| overall ($n = 29$) | 60 μg/L | ND | 8 μg/L | 0.16 | NC | |
| nonsmokers ($n = 5$) | 29 μg/L | ND | 17 μg/L[d] | 0.46 | 35 | ref *31* |
| smoker ($n = 1$) | 337 μg/L | ND | 111 μg/L | 0.25 | 305 | |
| overall ($n = 6$) | 35 μg/L | ND | 19 μg/L | 0.40 | 38 | |
| nonsmokers ($n = 60$) | 41.6 μg/L | ND | 8.7 μg/L[d] | 0.18 | 26.7[e] | ref *24* |
| smokers ($n = 60$) | 107.3 μg/L | ND | 15.0 μg/L | 0.13 | 64.1 | |
| nonsmokers ($n = 13$) | 26 μg/L | ND | 3 μg/L[d] | 0.12 | NC | ref *26* |
| occ. smokers ($n = 12$) | 56 μg/L | ND | 9 μg/L | 0.16 | NC | |
| smokers ($n = 13$) | 283 μg/L | ND | 20 μg/L | 0.07 | NC | |
| overall ($n = 38$) | 58 μg/L | ND | 8 μg/L | 0.14 | NC | |
| nonsmokers ($n = 47$) | 32 μg/L | NQ | 3 μg/L[d] | 0.07 | 16 | ref *22* |
| smokers ($n = 6$) | 184 μg/L | NQ | 10 μg/L | 0.06 | 74 | |
| nonsmokers ($n = 54$)[g] | 24.0 μg/L | 16.7μg/L | 3.8 μg/L[f] | 0.09 | 33.5 | this study |

[a] AAMA, AAMA-sulfoxide, and GAMA calculated as median concentration (μg/L) in urine. [b] Ratios of GA-derived metabolites to AA-derived metabolites excreted in human urine. [c] Median overall excretion of metabolites in urine calculated as AA-equivalents (μg/24 h). [d] GAMA quantitated without iso-GAMA. [e] Excretion of AA-equivalents (μg/24 h) calculated from mean excretion of AAMA and GAMA (μg) within 24 h (see reference). [f] GAMA includes both GAMA and iso-GAMA. [g] Median values calculated from 54 urine samples derived from the six individuals over 9 consecutive sampling intervals. [h] ND = not determined; NQ = not quantified; NC = not calculated.

metabolites arising from temporal factors within a day (e.g., time of sampling after food consumption or last urination) and across days (e.g., variable diets from day to day) are averaged out by monitoring over a prolonged period. The median values for AAMA and GAMA of the nonsmoking subjects were in good accordance with previously reported data (**Table 5**). However, AAMA-sulfoxide was demonstrated to be a major metabolite of AA excreted with urine of unintentionally exposed human subjects representing approximately 38% of the AA-metabolites excreted in urine. GAMA concentrations in the urine samples were much lower, indicating that, in contrast to rodents, the metabolic activation of AA to GA is only a minor pathway of AA-biotransformation in humans.

Since the participants of the study tried to avoid food known as major AA sources, metabolite concentrations in urine varied only marginally over time. The impact of foods with higher AA contents and the suitability of the method to detect changes in AA intake are indicated by the excretion profiles in subject M3, who had a meal of French fries on day three. This increase in AA exposure was rapidly reflected by an increase in AA metabolite excretion. An additional excretion of 22.3 μg AA equivalents (AAMA, AAMA-sulfoxide, and GAMA excreted in urine) was observed during day three as compared to days one and two, which is in good accordance with average contents of 28.3 μg AA in French fries per portion (*10*).

Assuming a continuous ingestion of acrylamide with the diet and constant excretion of metabolites with urine, the daily intake of AA corresponds directly to the daily excretion of urinary metabolites. The overall daily excretion with urine calculated as AA equivalents was 33.5 μg/24 h (range: 19.7−50.4 μg/24 h) for the six subjects. Accordingly, an intake of AA of 0.5 μg/kg b.w. per day (range: 0.25−0.81 μg/kg b.w. per day) was calculated in agreement with the estimated daily intake of 0.4 μg/kg b.w. AA (*10*).

## ABBREVIATIONS USED

AA, acrylamide; AAMA,*N*-acetyl-*S*-(2-carbamoylethyl)-L-cysteine; GA, glycidamide; GAMA, rac-*N*-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine; HILIC, hydrophilic interaction liquid chromatography; HPLC, high performance liquid chromatography; IS, internal standard; iso-GAMA, rac-*N*-acetyl-*S*-(1-carbamoyl-2-hydroxyethyl)-L-cysteine; LOD, limit of detection; LOQ, limit of quantification; MA, mercapturic acid; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RP, reversed phase; S/N, signal-to-noise ratio.

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