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Determination of the major mercapturic acids of acrylamide and glycidamide in human urine by LC–ESI-MS/MS

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Abstract

We developed a LC–MS/MS method for the quantitative determination of the mercapturic acid (MA) metabolites of acrylamide (AA) AAMA and of its oxidative metabolite glycidamide (GA) GAMA in urine samples from the general population. The method requires 4 mL of urine which is solid phase extracted prior to LC–MS/MS analysis. The metabolites are detected by ESI-tandem mass spectrometry in negative ionisation mode and quantified by isotope dilution. Detection limits ranged down to 1.5 μg/L urine for both AAMA and GAMA. The imprecision expressed as R.S.D. lay between 2% and 6% for both analytes (intra- and inter-assay). First results on a small group of 29 persons out of the general population ranged from 5 to 338 μg/L AAMA and <LOD to 45 μg/L GAMA in urine. Only in one urine sample GAMA could not be detected. With this sensitive, reliable and rapid method we can determine the internal exposure of the general population to acrylamide in terms of the mercapturic acids. Especially the determination of GAMA is of great toxicological importance because GA is the ultimate carcinogenic agent in AA metabolism. The method therefore provides better insight into the metabolism of acrylamide in humans and furthermore supports risk assessments.

Keywords: Acrylamide (AA); Glycidamide (GA); Biological monitoring; Mercapturic acids; Metabolites; *N*-Acetyl-*S*-(2-carbamoylethyl)-L-cysteine (AAMA); *N*-(*R*/*S*)-Acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA)

1. Introduction

Worldwide 200,000 t of acrylamide (AA) are produced each year [1]. It is used for a variety of purposes such as production of polymers (>90%) and formulation of cosmetics and body care products. Besides it is applied in the textile industry as well as in laboratories for gel chromatography, etc. [2]. During these processes some AA is released into the environment. In the U.S. for instance it was estimated that in 1994 1800 t of AA were emitted [3].

Of much greater concern for health aspects of the general population is AA as a constituent of normal diet. In 2002 Tareke et al. [4] found that AA is formed during the heating of carbohydrate-rich food. Therefore, foodstuff like french fries or potato chips showed peak concentrations of AA up to several mg/kg [5]. Also coffee, bread, cereals, etc. contain measurable amounts of AA. Apart from that AA is also a component of cigarette smoke [6].

To find AA in our daily diet has obtained considerable attention in the media and raised reasonable concern in the general population as AA is known to cause different kinds of cancer in animals [7–10] and also has some reproductive toxic effects [11–14]. AA has been classified as a probable human carcinogen by national and international expert committees [2,3,15,16]. Only recently the mutagenicity of acrylamide to mammalian cells was confirmed in the low doses range using mammalian cells [17,18].

Abbreviations: AAMA, *N*-acetyl-*S*-(2-carbamoylethyl)-L-cysteine; d_3-AAMA, *N*-acetyl-*S*-(2-carbamoyl-1,1,2-d3-ethyl)-L-cysteine; GAMA, *N*-(*R*/*S*)-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine; d_3-GAMA, *N*-(*R*/*S*)-acetyl-*S*-(2-carbamoyl-2-hydroxy-1,1,2-d_3-ethyl)-L-cysteine

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With respect to the risk assessment of the general population the determination of the internal exposure is of utmost importance.

Regarding the metabolic fate of AA in rodents it was shown that AA is partly oxidised to its epoxide glycidamide, in mice the involvement of Cytochrome P450 2E1 was clearly demonstrated [19]. Both AA and GA can further react with nucleophilic molecules in the body such as haemoglobin (hb) and glutathione (GSH) but also DNA (Fig. 1). Yet only GA-DNA adducts could be determined in vivo so far [20], which supports the assumption that GA is the predominant genotoxic agent [21].

Up to now hb-adducts of AA and its genotoxic metabolite GA have been successfully measured in human blood as biomarkers of the internal burden [22–25]. However, the analytical procedure is very time-consuming.

GSH-conjugates are excreted in urine as the according mercapturic acid after enzymatic split-off of glutamine and glycine and subsequent acetylation of the acid group (Fig. 1), As to whether enzymes like glutathione-*S*-transferases (GST) might be involved in the formation of GSH-conjugates of AA and GA could not be proved up to date. According to animal studies the most prominent metabolites excreted in urine were found to be the mercapturic acids of AA *N*-acetyl-*S*-(2-carbamoylethyl)-L-cysteine (AAMA) and of GA *N*-(*R*/*S*)-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA) [26,27], see Fig. 1. In humans AAMA could not be measured selectively up to now. Only one method in literature describes a HPLC method with fluorescence detection which lacks specificity for this analyte [28]. GAMA has not been determined at all in human urine up to now.

We therefore focussed on these two MA as parameters for the internal exposure and uptake of AA. For that purpose we developed a rapid analytical method for the simultaneous determination of both mercapturic acids in human urine. With this method it is now for the first time possible to determine specifically the mercapturic acid of AA and furthermore also of its metabolite GA. As GA is regarded to be mainly responsible for the genotoxic properties of AA the determination of especially GAMA formed in the human metabolism is supposed to provide hints on the genotoxic potency of AA in humans.

2. Experimental

2.1. Chemicals

N-Acetyl-*S*-(2-carbamoylethyl)-L-cysteine (AAMA) and its d_3-labelled analogue d_3-AAMA were synthesised in our laboratory from acrylamide (AA) respective acrylamide-2,3,3-d_3 (d_3-AA) and *N*-acetyl-L-cysteine (NAC) by Michael addition with sodium ethylate as a basic catalyst. Briefly 10 mmol NAC and 12 mmol AA (d_3-AA, respectively) were dissolved in ethanol and stirred at room temperature. 12 mmol sodium ethylate were dissolved in ethanol and then slowly added to the reaction mixture until the pH was about 8. The resulting precipitate was separated by filtration, washed with ethanol and vacuum-dried. The resulting products were characterised by mass spectrometry, ^{13}C and ^{1}H NMR. Purity was estimated to be >95% for both compounds.

N-(*R*/*S*)-Acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA) and its d_3-labelled analogue d_3-GAMA were custom-synthesised by Dr. H.-D. Gilsing of the "Institut für Dünnschichttechnologie und Mikrosensorik e.V." (IDM) in Teltow. In the first step acrylonitrile was reacted pH-controlled in aqueous solution to its epoxide. The second step was a nucleophilic attack of the SH-group of *N*-acetyl-L-cysteine at the epoxide leading to ring-opening. The reaction mixture contained GAMA which was purified by column-chromatography,

For d_3-GAMA acrylontrile-d_3-2,3,3 was epoxidised as described above. Then *N*-acetyl-L-cysteinemethylester was added followed by saponification of the methylester yielding d_3-GAMA. Purity for both compounds was >90%.

Acrylamide and sodium ethylate were purchased from Merck (Darmstadt, Germany). Acrylamide-2,3,3-d_3 was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Acetonitrile, water, methanol, ethanol (all of HPLC-grade), formic acid (98–100% GR for analysis) and hydrochloric acid (fuming 37%, GR for analysis) were purchased from Merck (Darmstadt, Germany). Ammonium formate was purchased from Fluka (Taufkirchen, Germany).

2.2. Instrumentation

The LC–MS/MS system consisted of a HPLC system Model HP 1100 with a quaternary pump, a vacuum degasser and an autosampler. The HPLC was directly coupled to a triple quadrupole mass spectrometer (Model Sciex API 2000, Applied Biosystems, Langen, Germany) equipped with a Turbo ion spray (TIS) source and a 10-port valve. Nitrogen was supplied by a system consisting of a compressor (Jun-Air Model 4000, Ahrensberg, Germany), membrane air dryer (Whatman Model 64-01, Maidstone, UK) and a nitrogen generator (Whatman Model 75-72).

2.3. Standard preparation

The stock solutions for the native standards were prepared by dissolving 10 mg AAMA and GAMA, respectively in 10 mL methanol (1.0 g/L). These stock solutions were stored at +4 °C in brown Teflon-capped glass vials until further use. The stock solutions were then diluted 1:10 and 1:100 resulting in the working solutions I (100 mg/L) and II (10 mg/L) which later served for the preparation of the standard solutions, see Section 2.4. The stock solutions for each internal standard (IS) were prepared by dissolving 5 mg in 50 mL methanol (100 mg/L). Both IS stock solution was then diluted 1:10 with methanol yielding the concerning internal standard solution for d_3-AAMA and d_3-GAMA (each 10 mg/L). All solutions

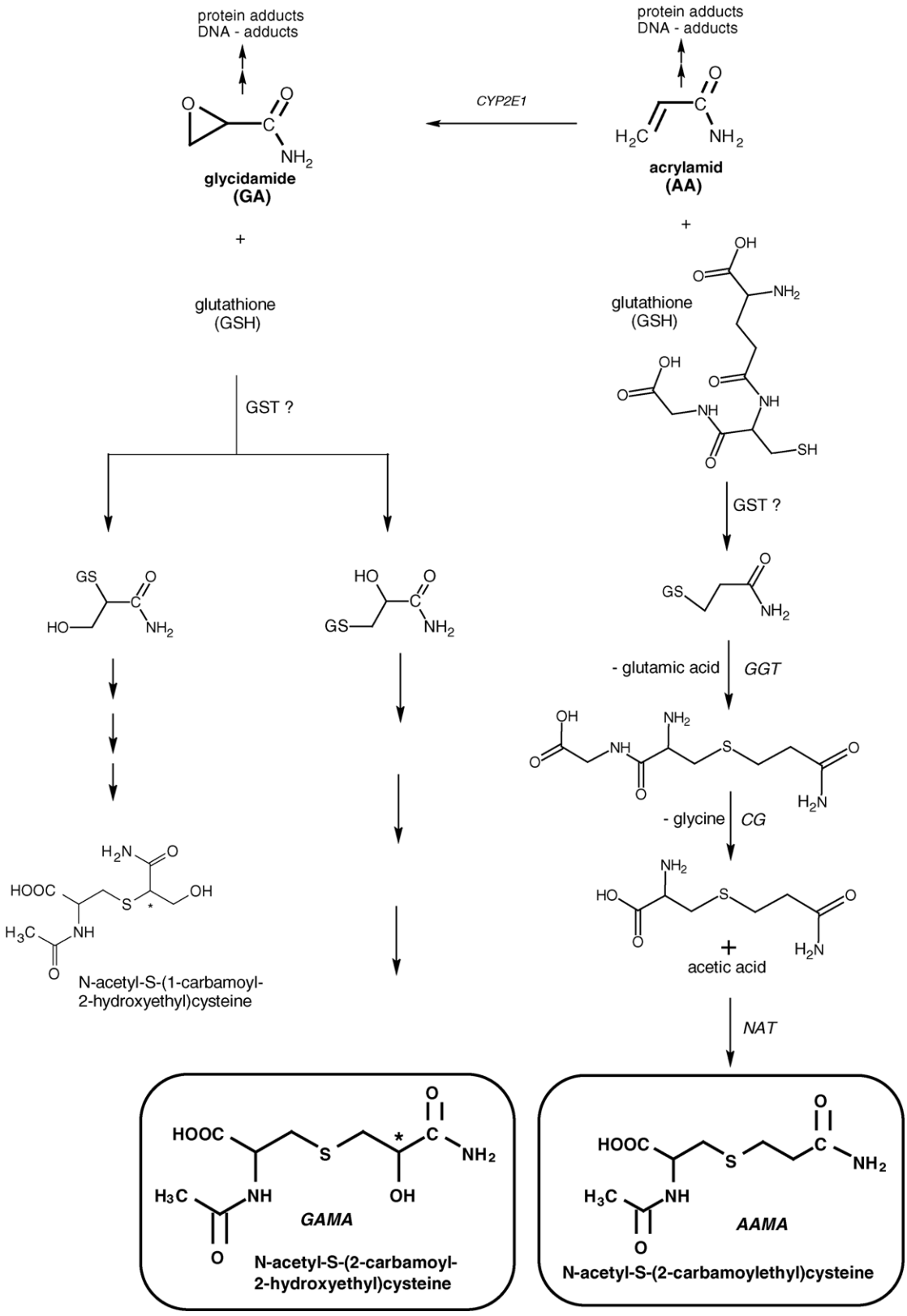

Fig. 1. Metabolic pathway of acrylamide with respect to the formation of mercapturic acids (in detail described for AAMA, in short for GAMA). The structures of the analysed metabolites are depicted in the boxes.

were finally stored at −20 °C in brown Teflon-capped glass vials, the stock solutions were stable for at least 12 months under these conditions.

2.4. Calibration procedure and quality control

From the standard working solutions I and II seven calibration standards were prepared by diluting these solutions (concentration range from 5 to 500 μg/L) with urine and water. The standards were processed as described in Section 2.5. Linear calibration curves were obtained by plotting the quotients of the peak areas of the AAMA and GAMA, respectively, and the corresponding d_3-internal standards as a function of the concentrations used. The coefficients of correlation for all calibration curves were higher than 0.999. These graphs were used to ascertain the unknown concentrations of the mercapturic acids in urine samples.

As there was no quality control material commercially available it had to be prepared in the laboratory. For a low-concentration (Q_{low}) and high-concentration (Q_{high}) control material native pool urine was spiked with AAMA and GAMA to yield concentrations of 16.2 μg/L (Q_{low}) and 162 μg/L (Q_{high}) for AAMA and 20.2 μg/L (Q_{low}) and 202 μg/L (Q_{high}) for GAMA, respectively. The pools were divided into aliquots and stored at −18 °C. For quality assurance one low- and one high-concentration control sample was included in each analytical series.

Within-series imprecision was determined by analysing the low- and high-concentration quality control urine eight times in a row. Between-day imprecision was determined by analysing the quality control samples on 6 (Q_{low}) and 10 (Q_{high}) different days.

Furthermore possible influences of urinary matrices was investigated by analysing eight different spiked individual urine samples with creatinine contents from 0.15 to 1.74 g/L. The mercapturic acid content of the non spiked specimens varied from subject to subject. Spiked specimens from persons of the general population (spiked concentration 54.1 μg/L AAMA and 67.5 μg/L GAMA) and the same specimens without the addition of the analytes were analysed.

2.5. Sample preparation

Urine samples were frozen until analysis. Prior to sample preparation frozen urine samples were thawed, equilibrated to room temperature and vortex-mixed. Then aliquots of 4 mL were transferred to 10 mL plastic tubes. Forty microlitres formic acid, 4 mL ammonium formate buffer solution (50 mmol/L, pH 2.5) and 30 μL of each d_3-internal standard solutions were added to the samples. The samples were vortex-mixed again and centrifuged at 3000 × *g* for 10 min. Meanwhile the SPE columns (Isolute ENV+, 100 mg, 10 mL XL from Separtis Grenzach-Wyhlen, Germany) were pre-conditioned with 4 mL methanol, 2 mL deionised water and 2 mL diluted formic acid (pH 2.5). Then 7.5 mL supernatant of the centrifugated sample solution were transferred onto the SPE columns. Afterwards the columns were washed with 2 mL diluted formic acid (pH 2.5) and 800 μL 10% (v/v) methanol in formic acid (pH 2.5). The analytes were then eluted with 1.7 mL 1% (v/v) formic acid in methanol. The elute solution was evaporated to dryness under a stream of nitrogen at 40 °C. The residue was then reconstituted in 1 mL 0.1% formic acid in deionised water (v/v).

One hundred microlitres of this solution were then injected into the LC–MS/MS system for quantitative analysis.

Urinary creatinine concentrations were determined according to Larsen [29].

2.6. LC–MS/MS analysis

After the clean-up and enrichment step on the SPE cartridge the analytes were injected onto a reversed phase HPLC column (Luna C8(2) 150 mm × 4.6 mm, 3 μm particle size from Phenomenex, Aschaffenburg, Germany). Injection volume was 100 μL and runtime was 30 min. Samples were eluted isocratically. The mobile phase consisted of 95% deionised water, 5% formic acid (1%, v/v) and 5% acetonitrile at a flow rate of 0.3 mL/min. A time controlled 10-port valve on the LC–MS/MS system was used to transfer the analyte fraction in a time slot of 4.5 min into the MS/MS system (runtime 7.0–11.5 min), before and after the slot the flow was directed into the waste to prevent the MS/MS system from avoidable contamination. A six-port valve of course would have been sufficient to perform this task. All steps were controlled by Analyst 1.1 Software from Perkin-Elmer. A guard column (C8 Octyl, 4 mm × 3 mm, Phenomenex) was placed in front of the analytical column to extend its lifespan. During routine measurements it was replaced after around 100 injections.

The MS/MS detection was performed on a Sciex API 2000 LC–MS/MS System. The ion source-dependent (Turbo ion spray) conditions were the same for all of the analytes with an electrospray needle voltage of −4000 V in the negative ion mode. Nitrogen as nebulizer and turbo heater gas (475 °C) was set at a pressure of 35 and 70 psi, respectively. The curtain gas was set to 30 psi. The collision gas (N_2) for the MS/MS mode at quadrupole Q2 was set to a flow of 3 (instrument units). Continuous flow injections of standard solutions for both analytes and internal standards were performed to establish the MS/MS operating conditions with the syringe pump system of the API 2000. For each analyte and internal standard two specific precursor and product ion combinations were monitored with one combination being used for quantification and the other for verification (predicted mass fragmentations see Fig. 2). MS/MS operating conditions in the MRM-mode for all parameters were as follows: resolution Q1, unit; resolution Q3, low; settling time, 0 ms; MR pause, 5 ms; scan time, 75 ms. Analyte specific parameters are shown in Table 1.

2.7. Study subjects

For a pilot study we investigated 29 spot urine samples from the general population as published elsewhere [30]. The study subject demographics were as follows: eight persons were male, 21 were female, aged between 26 and 41 and occupationally not exposed to acrylamide. Thirteen of them were smokers, 16 stated not to smoke. Creatinine lev-

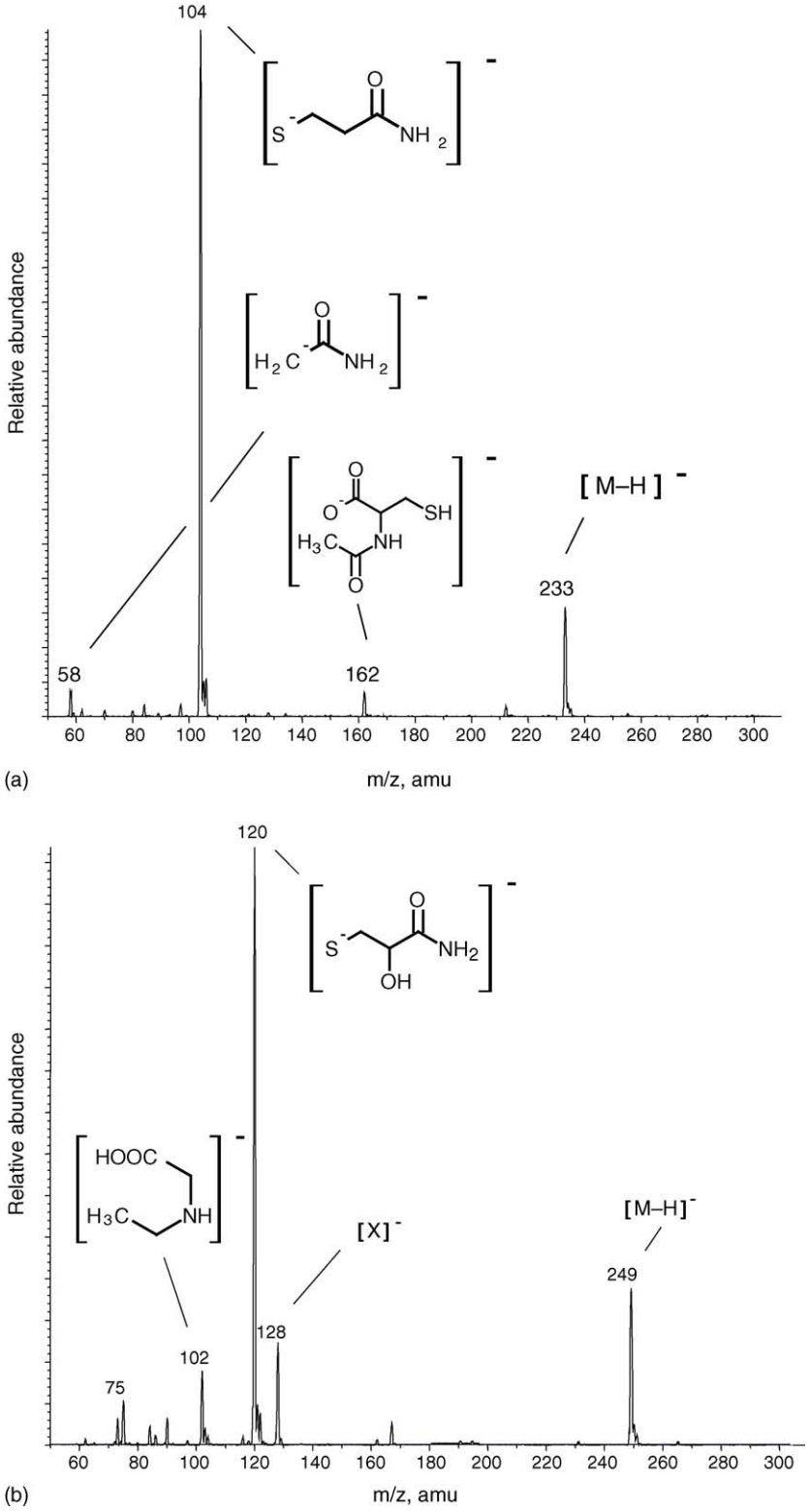

Fig. 2. ESI-negative Q1 mass spectrum of (a) AAMA; (b) GAMA; (c) d_3-AAMA and (d) d_3-GAMA with the predicted structures of fragments. $[X]^-$: unknown fragment.

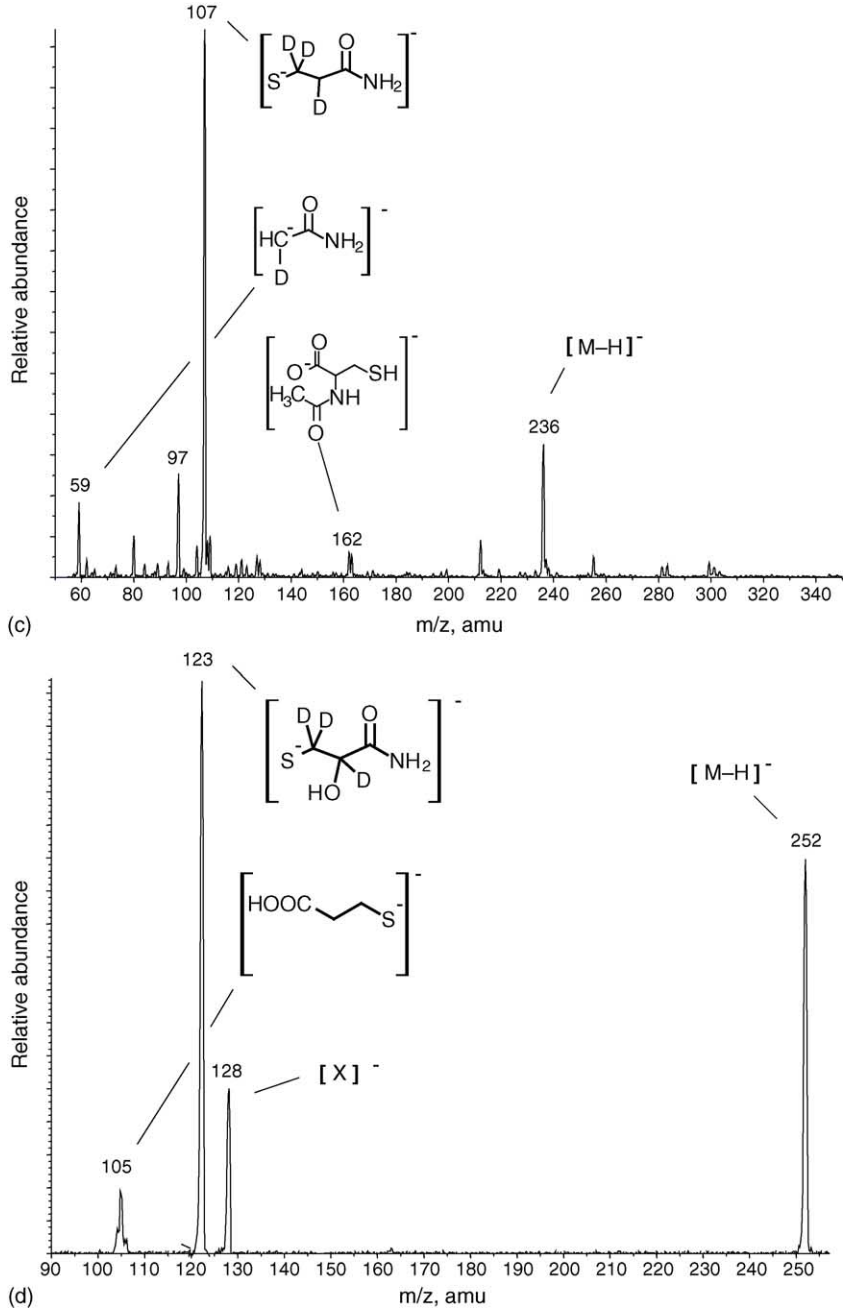

Fig. 2. (*Continued*).

els were in the range from 0.15 to 1.70 g/L. Results see Section 3.7.

3. Results and discussion

3.1. General considerations

Motivation for developing this method was to cover the most important urinary metabolites of AA resulting from the reductive (direct reactions of AA) and the oxidative (reactions of GA) metabolic pathway in one analytical procedure.

In order to build a fast, economic and reliable method we tried to apply novel HPLC on-line cleanup and enrichment column-switching techniques, however, the analytes turned out to be too polar for the commercially available online-phases (Merck RAM ADS RP-4, RP-8 and RP-18, Oasis HLB as well as several other short analytical columns esp. suitable for polar compounds, e.g. Phenomenex Capcell Pak MG and AQ). In general retention was too low or not existing at all so that we decided to use an offline solid phase extrac-

Table 1
MRM-parameters for each specific precursor and product ion combination

Analyte	Precursor ion (Q1)	Product ion (Q3)	Retention time (min)	DP (V)	FP (V)	EP (V)	CE (V)
AAMA	233	104*	9.4	−36	−350	7.5	−16
	233	58	9.4	−36	−310	7.0	−46
d_3-AAMA	236	107*	9.4	−1	−290	11.0	−16
	236	59	9.4	−6	−280	10.5	−48
GAMA	249	120*	8.8	−6	−320	11.5	−26
	249	102	8.8	−11	−270	10.5	−24
d_3-GAMA	252	123*	8.8	−16	−350	11.0	−18
	252	105	8.8	−16	−310	11.5	−30

The combinations used for quantification purposes are marked with an asterisk (*). *The precursor–product ion combinations used for quantification. DP: declustering potential; FP: focussing potential; EP: entrance potential; and CE: collision energy.

tion and subsequent liquid chromatography combined with state-of-the-art MS/MS detection. The use of the highly sensitive and selective ESI-MS/MS-detection ensured a smooth performance.

In 1994 Calleman et al. [31] had measured and tried to quantify AAMA in human urine of 41 workers heavily exposed to AA and acrylonitrile (ACN) and of 10 controls. They applied the method of Wu et al. [28] which turned out to be not specific for AAMA. Analytes were determined after hydrolisation as carboxyethylcysteine (CEC). CEC is, e.g. also formed after hydrolisation of cyanoethylcysteine (CEMA), a mercapturic acid of acrylonitrile which is excreted in urine after exposure to ACN. Therefore, the determination of CEC as a biomarker leads to an overestimation of the AA-exposure in the case of a simultaneous ACN-exposure for instance by tobacco smoking or occupational exposure.

For the first time we now present a highly selective and specific method measuring mercapturic acids of acrylamide and glycidamide simultaneously.

3.2. Sample preparation

In preparation for the SPE-procedure the frozen urine samples were thawed and centrifuged in order to precipitate cryophilic proteins and particulate matter. We then applied an offline SPE sample preparation step for clean-up and concentration purposes. Several commercially available cartridges were tested like conventional silica-based C18 material, anion exchange material as well as resin-based RP-materials. “Isolute ENV+” is a modified polyvinylstyrol-divinylbenzene resin (with additional hydroxy-groups) which was designed to cover a wide range of analyte polarity and appeared to be the most suitable cartridge for our purposes. This SPE-phase enabled us to extract and enrich both analytes out of the matrix as an effective cleanup.

3.3. Liquid chromatography

After the offline SPE procedure the aliquots of the analyte containing solutions were injected onto the analytical column and led into the ion from 7.0 to 11.5 min by the 10-port switching valve of the Sciex API 2000 mass spectrometer. Mobile phase before and after this fraction was led directly into the waste to prevent the ion source from needless contamination. The isocratic solvent was optimised to a 0.05% aqueous solution of formic acid and acetonitrile (90:10, v/v).

The chromatographic separation was optimised to cut off disturbing substances out of the urinary matrix. Best peak shapes and separation properties were obtained on a Luna C8 column (chromatograms of different unspiked urine samples shown in Figs. 3 and 4). Fig. 5 shows the quantifier ion traces of each internal standard for AAMA and GAMA, respectively, of a processed native urine sample (no internal standard added). The other tested silica- and resin-based columns showed too low retention times or unsatisfactory peak shapes so that this RP 8 column was our first choice.

3.4. Mass spectrometry

Urban et al. achieved excellent results in the detection of two mercapturic acids of 1,3-butadiene and several other mercapturic acids applying atmospheric pressure chemical ionisation (APCI) [32] with limits of detection (LOD) in the lower μg/L range. We therefore tested the APCI-source and compared it to electrospray ionisation (ESI) on the API 2000. The APCI turned out to produce lower signal to noise ratios than the ESI although the intensity of the signals were significantly higher. We got excellent results with ESI in the negative mode in terms of low disturbance from coeluting substances and only few interferences as well as high signal to noise ratios without extensive co-optimisation of related APCI parameters. Compound specific mass spectrometer parameters were optimised for each compound automatically by the Quantitative Optimisation Wizard of the Sciex Analyst™ software. The source specific parameters were optimised manually for the LC conditions used during analysis depending on flow rate and composition of the mobile phase. Q1 ESI negative ion mass spectra with tentative fragment structures

for AAMA, GAMA, d_3-AAMA and d_3-GAMA are shown in Fig. 2a–d. The chosen precursor ions for the MS/MS fragmentation of all analytes were $[M-H]^-$. At least two product ions were monitored for each analyte ensuring maximum selectivity. With a scan time for each fragmentation of 75 ms six precursor–product ion combinations were registered simultaneously in the multiple reaction monitoring (MRM) mode of the API 2000. All combinations were registered within the same time-window. MRM-parameters are illustrated in Table 1. The identity of each analyte peak was confirmed by two precursor–product ion combinations and by matching its retention time with its d_3-labelled internal standard.

For quantification the peak area ratio of analyte to d_3-internal standard was used. No unlabelled isotope fragments were detectable in the labelled standards even at high con-

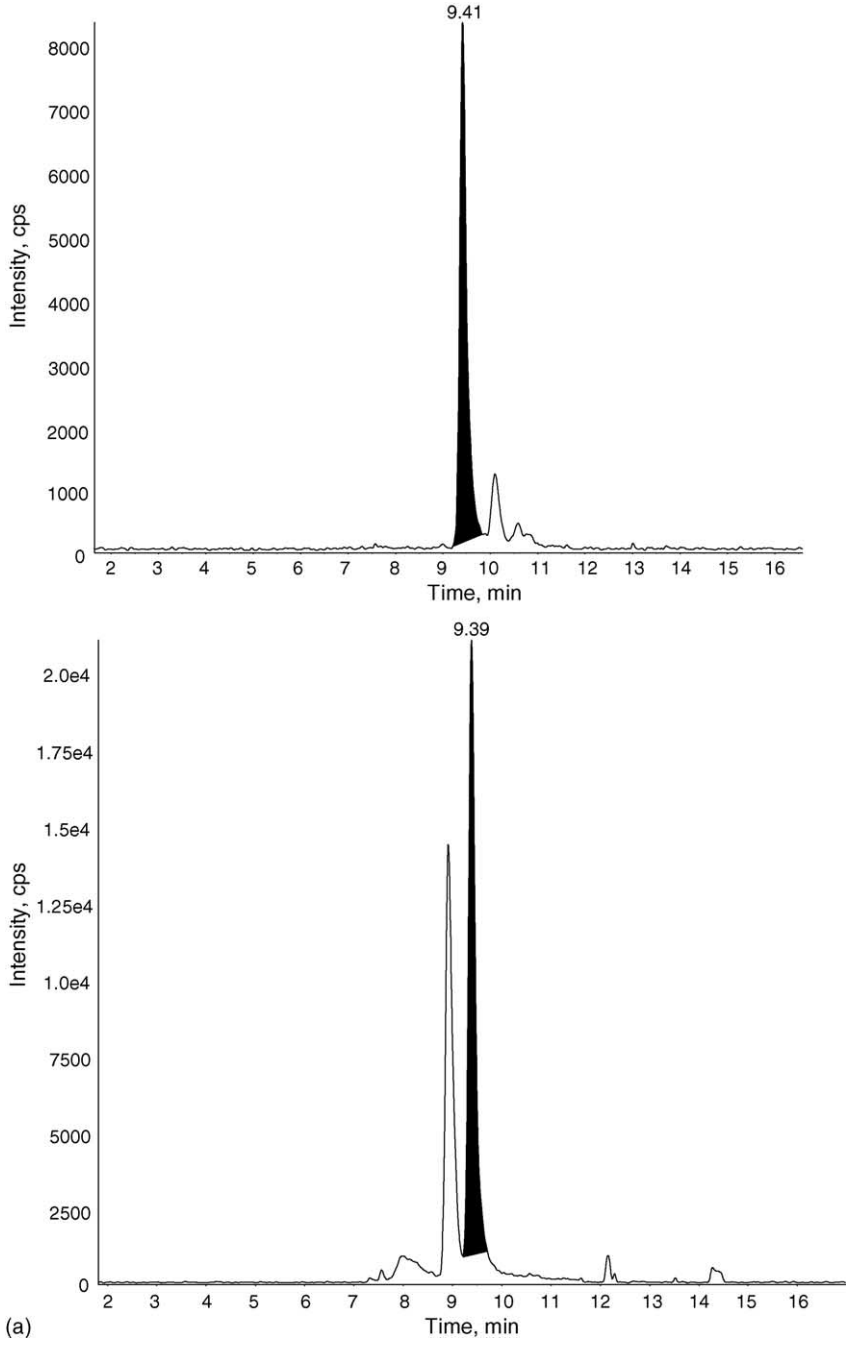

Fig. 3. Chromatogram of a processed native (not spiked) non-smoker urine sample (mind the different scales on the *y*-axis). (a) The quantifier precursor–product ion traces are given above for AAMA (233 → 104) and below for d_3-AAMA (236 → 107). (b) The quantifier precursor–product ion traces are given above for GAMA (249 → 120) and below for d_3-GAMA (252 → 123).

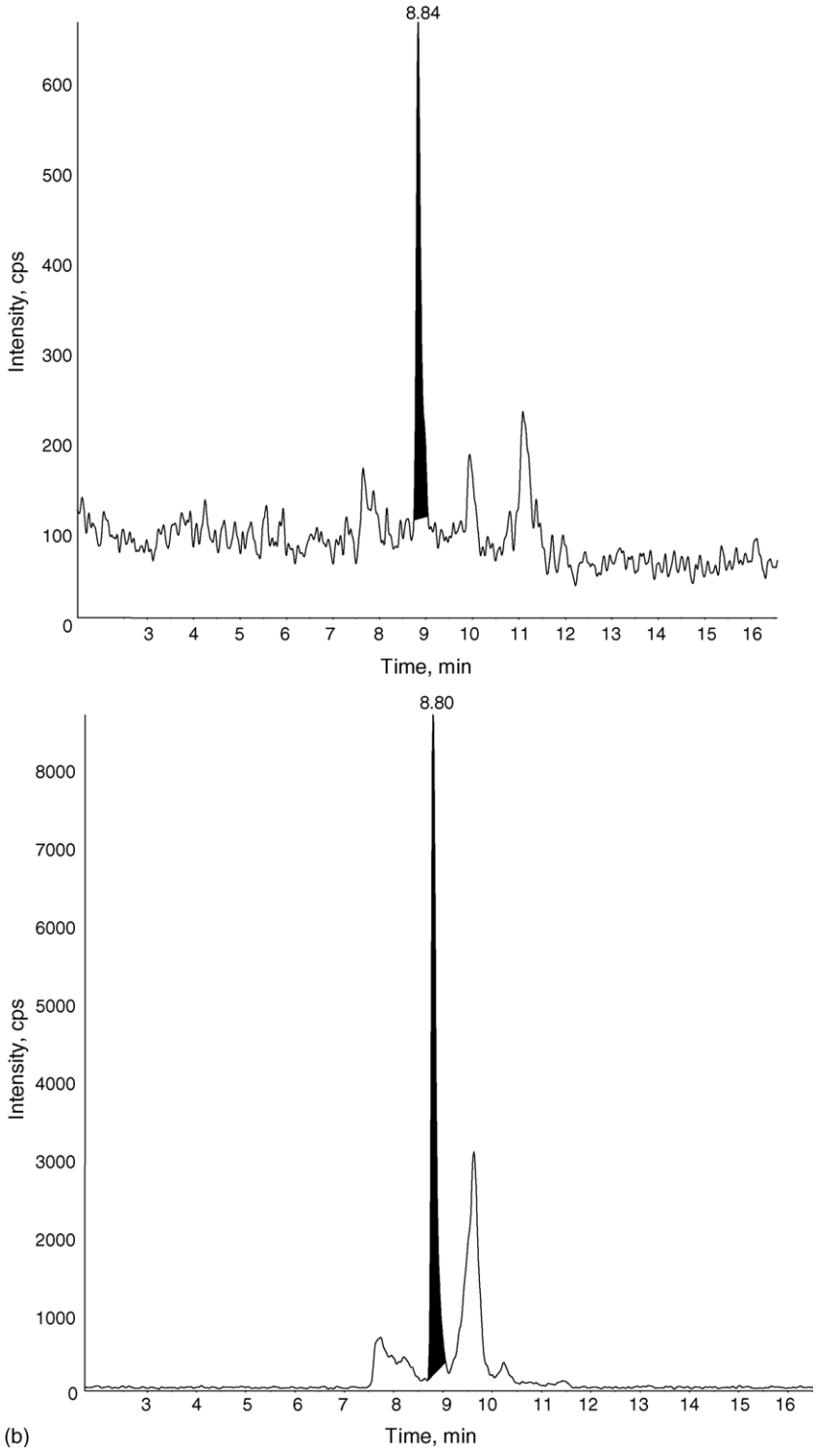

Fig. 3. (*Continued*).

centrations (10 mg/L) and after going through the analytical process. No D–H isotope exchange interfering with the analytical precision was observed.

In a few cases ionisation was suppressed resulting in loss of sensitivity. This is probably due to coeluting substances in the urinary matrix. Since quantification was based on isotope dilution this variation in absolute signal intensity did not significantly deteriorate quantitative accuracy unless ionisation was dramatically suppressed in very few cases (see Section 3.6.1), e.g. regarding the recovery of only 72% in

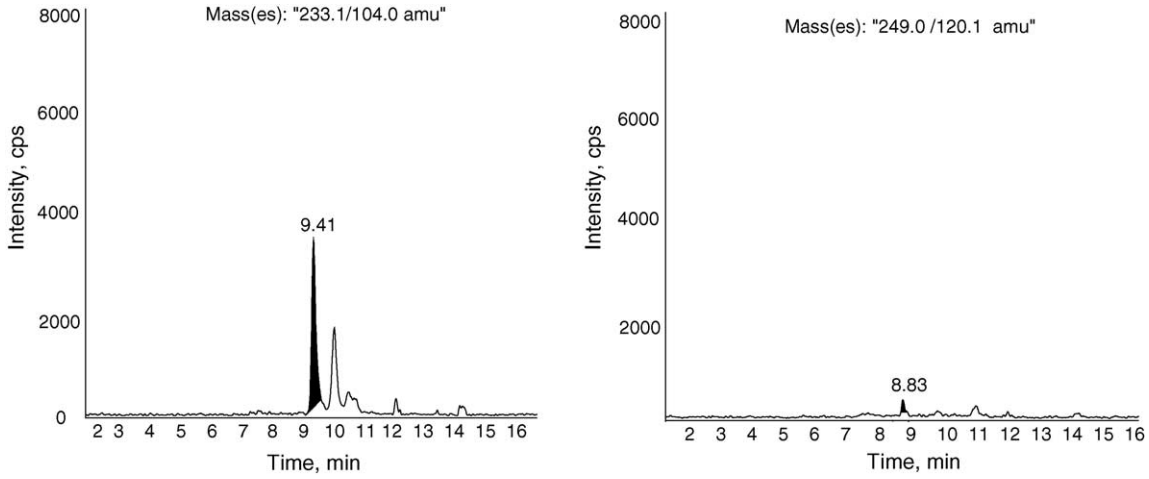

Fig. 4. Chromatogram of a processed "blank" low-burdened urine sample (used for quality control material), left side: quantifier ion trace of AAMA (233 → 104, concentration: 16 μg/L), right side: quantifier ion trace of GAMA (249 → 120, concentration: <LOQ).

one case of the experiment with eight individual spiked urine samples.

3.5. Calibration graphs

Calibration graphs were obtained by analysing solutions of the standards in urine and water in the concentration range of 5–500 μg/L. As there was no observable difference in calibration with water and urine we conducted further calibration in water only.

All calibration curves were linear within the given concentration range (and even up to 1000 μg/L, which was tested separately) and produced linear correlation coefficients all greater 0.999. This wide working range was necessary to cover the highly variable levels of the different mercapturic acids in human urine.

3.6. Reliability of the method

3.6.1. Reproducibility and accuracy

In order to assess the within-series imprecision, control materials Q_{low} and Q_{high} were analysed eight times in a row. Q_{low} contained about 15–20-times the LOD for each analyte, Q_{high} contained about 150–200-times the LOD for each analyte and both were prepared from pooled urine. For Q_{low} relative standard deviations were 3% for AAMA and 6% for GAMA, for Q_{high} the relative standard deviations were 2% for AAMA and 5% for GAMA demonstrating good reproducibility of this method over a wide concentration range down to trace levels.

The relative standard deviation of the between-day imprecision was determined on 6 different days for Q_{low} with 5% both for AAMA and GAMA. For Q_{high} it was determined on

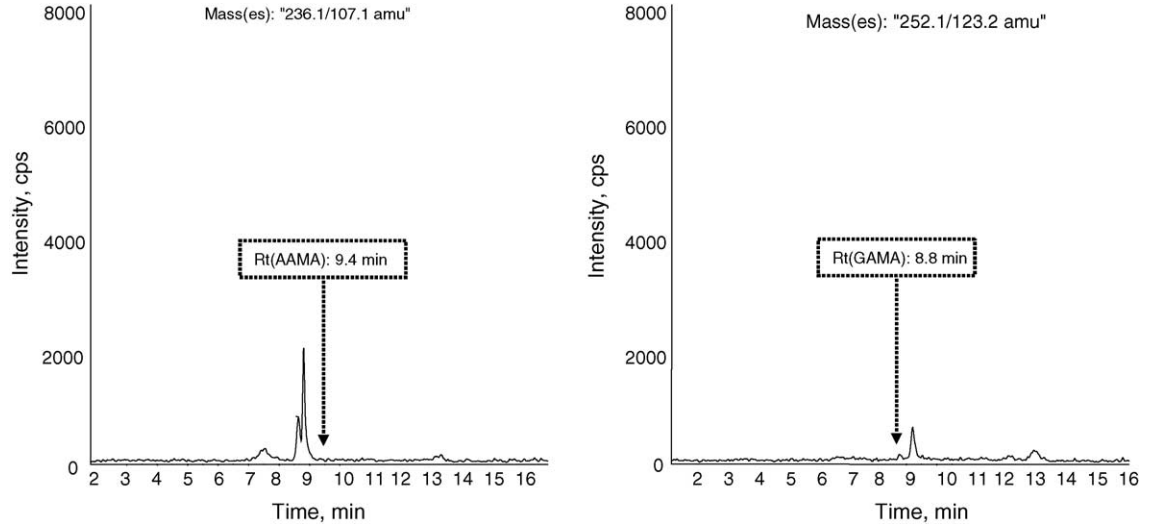

Fig. 5. Chromatogram of a processed urine sample without addition of internal standard, left side: quantifier ion trace of d_3-AAMA (236 → 107), right side: quantifier ion trace of d_3-GAMA (252 → 123).

Table 2
Quality control data and quantification limits of this method for the determination of AAMA and GAMA in human urine

	Imprecision						Accuracy		LOQ (μg/L)
	Q_{low}			Q_{high}			Relative recovery (%)		
	n	*c* (μg/L)	R.S.D. (%)	*n*	*c* (μg/L)	R.S.D. (%)	Mean	Range	
AAMA									
Intra-day	8	16.2	2.9	8	162	2.2	99	92–109	5
Inter-day	6	16.2	4.9	10	162	3.8	96	88–113	5
GAMA									
Intra-day	8	20.2	6.2	8	202	5.3	101	89–115	5
Inter-day	6	20.2	4.8	10	202	6.0	101	93–112	5

Table 3
Precision and accuracy data for the determination of AAMA and GAMA in eight different urine specimen matrices (test of robustness/matrix dependency)

	n	Native content (μg/L)		Spiked (μg/L)	Relative recovery (%)	
		Mean	±R.S.D.		Mean	Range
AAMA						
Individual urine specimens	8	53	25	54.1	97	81–110
GAMA						
Individual urine specimens	8	12	7	67.4	95	72–113

10 different days with 4% for AAMA and 6% for GAMA. All data are presented in Table 2.

Imprecision was also calculated based on the eight individual urine samples from the recovery experiments described below. The relative standard deviations for the parameters at the spiked concentrations were 8–13%.

As no certified reference material was commercially available, accuracy was checked by an additional special recovery experiments (see Table 3). Eight different urine specimens were spiked with 54.1 μg/L AAMA and 67.4 μg/L GAMA. The urine specimens were selected to reflect a composition as different as possible. As an indicator we use the creatinine content which for the eight urine specimens ranged from 0.15 to 1.70 g/L. In that way we can also check for a possible influence of the complex biological matrix on the analytical result. Native concentrations were found to be 21–91 μg/L for AAMA and 5–24 μg/L for GAMA. Relative recoveries were 97% (mean) for AAMA and 95% (mean) for GAMA in the range from 72% to 113% for both analytes and all specimens (see Table 3). So even for very different composed urine samples good recovery rates were obtained demonstrating the robustness and again the good accuracy of this method.

The reliability of the method was also proven by the good correlation of both analytes as shown before ($r = 0.879$) [30].

3.6.2. Detection limit and quantification limit

The limit of detection, defined as a signal-to-noise ratio of three for each quantitier ion trace were estimated to be 1.5 μg/L for both analytes. The limits of quantification (LOQ), defined as a signal-to-noise ratio of 10, were therefore estimated to be 5.0 μg/L.

3.6.3. Sources of error

The developed LC–MS/MS method has proven to be robust and reliable. The guard column was replaced after 100 real life sample injections.

3.7. Results of biological monitoring

The results of the biomonitoring of the 29 persons not occupationally exposed to acrylamide are summarised in Table 4 as published elsewhere [30]. Urine samples contained AAMA and GAMA in different concentrations differing 15-(GAMA) to 100 (AAMA)-fold from subject to subject. Fig. 3 shows a representative chromatogram of a processed non-smoker spot urine sample. The results suggest the exposure of the general population to acrylamide is not negligible and in some cases unexpectedly high. This exposure is very likely caused by dietary intake and also clearly depended on the

Table 4
Results of biological monitoring ($n = 29$): urinary AA-metabolites in human urine

Collective	AAMA (μg/L urine)	GAMA (μg/L urine)
Overall ($n = 29$)		
Median	60	8
Range	<LOD–338	<LOD–45
Smokers ($n = 13$)		
Median	127	19
Range	17–338	<LOD–45
Non smokers ($n = 16$)		
Median	29	5
Range	<LOD–83	<LOD–14

smoking habit (see Table 4), of minor importance may be the use of cosmetics or other environmental sources.

4. Conclusions

We have developed a reliable, easy and robust analytical procedure for the determination of two mercapturic acids of acrylamide and glycidamide, respectively, in human urine samples.

Within- and between-day imprecision is very good, even at low concentrations but also over a wide concentration range. The clean-up procedure and chromatographic separation are efficient so that almost no interfering effect of the matrix on the analytical results was observed. Consequently the analytical background interference is very low yielding 1.5 μg/L as limits of detection.

It is the first time that the mercapturic acids of AA and GA, respectively, AAMA and GAMA could be analysed specifically in human urine. Even more important is the fact that the mercapturic acid of GA (GAMA) can now be determined in human urine, as GA is regarded to be the ultimate carcinogenic species of AA.

Our developed method is suited for determining levels of these acrylamide-derived metabolites over a wide concentration range and can therefore be applied in the field of environmental medicine for assessing the body burden of the general population but also of occupationally exposed subjects. We proved that there is a considerable background exposure to acrylamide in the general population with a considerably high degree of variation between different individuals.

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