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

Melanie Isabell Boettcher, Jürgen Angerer *

Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, University of Erlangen-Nuremberg, Schillerstrasse 25, Erlangen D-91054, Germany

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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

* Corresponding author. Tel.: +49 9131 85 22374; fax: +49 9131 85 26126.
E-mail address: Juergen.Angerer@ipasum.imed.uni-erlangen.de (J. Angerer).

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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