Chemico-Biological Interactions 237 (2015) 38–46

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Synthesis, characterization and analysis of the acrylamide- and glycidamide-glutathione conjugates

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ARTICLE INFO

Article history:
Received 28 July 2014
Received in revised form 17 March 2015
Accepted 4 May 2015
Available online 15 May 2015

Keywords:
Acrylamide
Glycidamide
Glutathione conjugates
LC–ESI-MS/MS

ABSTRACT

Acrylamide (AA) is reported present in high-temperature-processed food and classified as a possible human carcinogen. *In vivo* metabolic activation of AA by CYP 2E1 to glycidamide (GA) may play an important role on AA carcinogenicity. AA and GA can be detoxified by glutathione-S-transferase to form AA and isomeric GA glutathione conjugates (AA-, GA2- and GA3-GSH, respectively), which can be further metabolized to mercapturic acids (MAs). Although many studies analyzed MAs in urine of rodents and humans, few studies have characterized and analyzed the GSH conjugates. The objectives of this study were to synthesize, purify, and characterize AA-GSH, GA2-GSH, GA3-GSH, ($^{13}C_3$)-AA-GSH, ($^{13}C_3$)-GA2-GSH, and ($^{13}C_3$)-GA3-GSH to develop an isotope-dilution liquid chromatography tandem mass spectrometry (LC–MS/MS) method to analyze AA- and GA-GSHs in blood of rats treated with AA. After purification and characterization of these conjugates, the LC–MS/MS method was developed and validated. This method reveals a limit of detection ($S/N = 3$) at 0.017 and a limit of quantitation ($S/N = 10$) at 0.05 ng/mL of serum for AA-GSH, 0.075 and 0.25 ng/mL for GA2-GSH, and 0.15 and 0.5 ng/mL for GA3-GSH. Analyzed with this method, AA-GSH, GA2-GSH and GA3-GSH were 1651.1 ± 374.5, 18.4 ± 6.3 and 75.3 ± 31.3 ng/mL in blood of male rats at 2 h after treatment with 5 mg/kg bw of AA by ip injection. These results showed that the LC–MS/MS method was successfully developed to analyze AA-GSH, GA2-GSH and GA3-GSH with satisfying sensitivity of AA and GA which were conjugated by glutathione *in vivo*.

1. Introduction

Acrylamide (AA) is a widely-used chemical in industries, and in products used for water treatment, oil drilling, papermaking and mineral processing. AA is neurotoxic to humans and rodents, and classified as a probable carcinogen to human by International Agency of Research on Cancer [1]. AA is also present in high-temperature-processed foods from the reaction between asparagine and reducing sugars, so-called "the Maillard browning reaction" [2]. AA is also present in tobacco smoking [3]. Average daily intake of AA to the general public was estimated in the range of 0.3–0.8 μg/kg/day by World Health Organization [4], and 0.31–1.1 μg/kg/day for adults, 0.43–1.4 μg/kg/day for adolescents, and 0.7–2.05 μg/kg/day for children in Europe [5]. The potential health effects have been of great concerns due to daily intakes of AA through food consumption.

Upon absorption, AA can be metabolically activated by cytochrome P450 2E1 (CYP2E1) to glycidamide (GA) [6], which is critical to AA genotoxicity [7–11]. GA can be spontaneously or enzymatically hydrolyzed to glyceramide [12]. Both AA and GA can also be detoxified through enzymatic (by glutathione-S-transferase, GSTs) or non-enzymatic pathways to

Abbreviations: AA, acrylamide; AAMA, N-acetyl-S-(2-carbamoylethyl)-cysteine; COSY, correlation spectroscopy; EH, epoxide hydrolase; GA, glycidamide; GAMA, N-acetyl-S-(2-hydroxy-2-carbamoylethyl)-cysteine; GSH, glutathione; GST, glutathione-S-transferase; HESI, heated electro spray ionization; HMBC, heteronuclear multiple-bond correlation spectroscopy; HSQC, heteronuclear single-quantum correlation spectroscopy; LOD, limit of detection; LOQ, limit of quantification; MAs, mercapturic acids.

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http://dx.doi.org/10.1016/j.cbi.2015.05.002
0009-2797/

form AA- and GA-glutathione conjugates (AA-GSH, GA2-GSH and GA3-GSH), respectively (see Scheme 1). The AA- and GA-GSH would be further metabolized by γ-glutamyl-transpeptidase, dipeptidases, and N-acetyltransferase to N-acetyl-S-(2-carbamoyl ethyl)-cysteine (AAMA) and N-acetyl-S-(2-hydroxy-2-carbamoyle thyl)-cysteine (GAMA) and excreted through urine. These mercapturic acids have been well studied to serve as biomarkers for current AA exposures and to imply AA metabolism *in vivo* [12–17].

Analysis of the AA- and GA-glutathione conjugates will further provide direct evidences of detoxication of AA and GA *in vivo* and can serve biomarkers to study interspecies differences in AA detoxication. Therefore, the objective of this study was to synthesize, purify, and characterize the AA- and GA-GSH and to develop an isotope-dilution-LC–MS/MS method to analyze these conjugates in blood of rats treated with AA. This is the first study to simultaneously quantify AA- and GA-GSH in order to clarify the kinetics of AA detoxication in rats.

2. Materials and methods

2.1. Chemicals

AA (99.9%, 25 g) was purchased from Alfa Aesar (Ward Hill, MA). GA (≧96.0%, 100 mg), L-glutathione (Reduced, ≧98.0%, 1 g), sodium hydrogen carbonate (99.7%, 1 kg) and formic acid (≧98.0%, 500 mL) were purchased from Sigma–Aldrich (St. Louis, MO). ($^{13}C_3$)-AA (chemical purity: 99.0%; isotope purity: 98.0%) was from Sigma–Aldrich (St. Louis, MO) and ($^{13}C_3$)-GA (chemical purity: 97.0%; isotope purity: 98.0%) from Toronto Research Chemicals (North York, Canada). Acetonitrile (≧99.9%, 4 L) was supplied by Merck (Darmstadt, Germany). De-ionized water was prepared by the Milli-Q system (Billerica, MA).

2.1.1. Synthesis, purification and characterization of AA-GSH adduct

AA (35 mM, 2.5 mg in 1.0 mL of H_2O) and GSH (35 mM, 10.8 mg in 1.0 mL of H_2O) were added into 8 mL $NaHCO_{3(aq)}$ buffer (50 mM, pH = 9) and stirred at 45 °C for 24 h. AA-GSH was purified by using a semi-preparative C18 column (5 μm, 10 × 250 mm, Phenomenex Co., Torrance, CA) with an isocratic elution consisting of 0.1% formic acid in H_2O (v/v); detection was performed with a HPLC-UV detector (210 nm, Jasco UV-975, Easten, MD). The mobile phase was delivered at a flow rate of 2 mL/min by a binary LC pump (Jasco PU-980). The fraction containing AA-GSH was collected with a fraction collector (CHF121SA, Toyo Seisakusho Kaisha, Ltd, Japan), pooled, and dried under vacuum. The residue was dissolved in formic acid/ H_2O (0.1%, v/v) and characterized with a triple quadruple tandem mass spectrometer (Thermo TSQ, Ringoes, NJ) by infusion at a flow rate of 10 μL/min. The MS/MS was operated with a HESI source under positive ion mode. Fragmentation of AA-GSH is shown in Fig. 1a.

Parts of the purified standards were dissolved in deuterium oxide and further characterized with ^{1}H NMR (Bruker AVIII 500 MHz FT-NMR, Instrumentation Center, NTU). The NMR spectrum shows that δ [ppm] = 2.06–2.10 (m, 2H, c-H), 2.43–2.51 (m, 2H, d-H), 2.50 (t, J = 6.8 Hz, 2H, 2-H), 2.70–2.78 (m, 2H, 3-H), 2.82 (dd, $J_{j',\ j''}$ = 14.0 Hz, $J_{j',\ f}$ = 9.0 Hz, 1H, j′-H), 3.00 (dd, $J_{j',\ j''}$ = 14.0 Hz, $J_{j'',\ f}$ = 5.0 Hz, 1H, $_{j''}$-H), 3.72 (t, J = 6.2 Hz, 1H, b-H), 3.87 (s, 2H, h-H), 4.50 (dd, $J_{f,\ j'}$ = 8.5 Hz, $J_{f,\ j''}$ = 5.0 Hz, 1H, f-H).

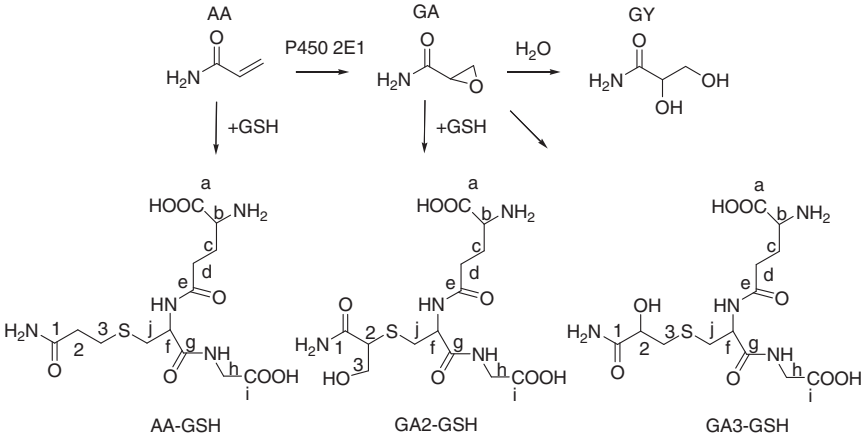
($^{13}C_3$)-AA-GSH was synthesized by following the identical procedures of AA-GSH synthesis, from the reaction of ($^{13}C_3$)-AA with GSH. The MS/MS spectrum of purified ($^{13}C_3$)-AA-GSH showed the same fragmentation pattern as AA-GSH. The precursor ion was m/z 382 ([M+H]$^+$). The product ions were m/z 253, m/z 307 and m/z 107.

2.1.2. Synthesis, purification and characterization of GA2-GSH and GA3-GSH adduct

GA (17.4 mg, 200 mM in H_2O, 1 mL) and GSH (61.5 mg, 200 mM in H_2O, 1 mL) were added into 8 mL $NaHCO_{3(aq)}$ buffer (50 mM, pH = 9) and stirred at 45 °C for 24 h. GA2-GSH and GA3-GSH were isolated from the product mixture by using the identical HPLC settings as for the purification of AA-GSH. Isocratic elution (0.1% formic acid, (v/v, %) was employed for the separation. The fraction with the peaks at retention times (t_R) of 22 and 28 min were collected and dried under vacuum. Diastereomers of GA3-GSH were further purified as model compounds for characterization with NMR.

MS/MS analysis was operated under the identical conditions with those used to characterize AA-GSH. Fragmentations of GA2-GSH and GA3-GSH are presented in Fig. 1b and c correspondingly.

The purified GA2-GSH and GA3-GSH were dissolved in deuterium oxide and further characterized by ^{1}H NMR, ^{13}C NMR, HSQC, HMBC and H,H-COSY (Bruker AVIII 500 MHz FT-NMR, Instrumentation Center, NTU). The NMR data for GA2-GSH were as follows: ^{1}H NMR (500 MHz, D_2O): δ [ppm] = 2.07–2.10 (m, 2H, c-H), 2.44–2.49 (m, 2H, d-H), 2.87–3.11 (m, 2H, j-H), 3.49–3.51 (m, 1H, 2-H), 3.70–3.78 (m, 2H, 3-H), 3.80 (s, 2H, h-H), 4.52–4.55 (m, 1H, f-H). ^{13}C NMR (500 MHz, D_2O): δ [ppm] = 26.1 (CH_2-c), 31.3 (CH_2-d), 32.5; 32.7 (CH_2-j), 42.4 (CH_2-h), 50.0; 50.2 (CH-2), 53.0; 53.2 (CH-f), 54.0 (CH-b), 61.5(CH_2-3), 172.00 (C-g), 173.83 (C-a), 174.77 (C-i), 174.85; 174.89 (C-e), 175.6; 175.8 (C-1).



Scheme 1. AA metabolism *in vivo*. AA is metabolized to GA by P450 2E1. AA and GA can react with GSH to form AA-GSH, and GA2-GSH and GA3-GSH, respectively.