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Quantification of urinary *N*-acetyl-*S*-(propionamide)cysteine using an on-line clean-up system coupled with liquid chromatography/tandem mass spectrometry

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Acrylamide has been reported to be present in high-temperature processed foods and normal processed food intake could lead to significant acrylamide exposure. Acrylamide *in vivo* can be conjugated with glutathione in the presence of glutathione transferase. This conjugation product is further metabolized and excreted as *N*-acetyl-*S*-(propionamide)cysteine (NASPC) in the urine. NASPC could be considered a biomarker for acrylamide exposure. The objective of this study was to develop a highly specific, rapid and sensitive method to quantify urinary NASPC, serving as a biomarker for acrylamide exposure assessment. Isotope-labeled [$^{13}C_3$]NASPC was successfully synthesized and used as an internal standard. This urine mixture was directly analyzed using a newly developed liquid chromatographic/tandem mass spectrometric method coupled with an on-line clean-up system. The detection limit for this method was estimated as <5 μg l^{-1}(0.4 pmol) on-column. The method was applied to measure the urinary level of NASPC in 70 apparently health subjects. The results showed that the NASPC urinary level was highly associated with smoking. Smokers had a significantly higher urinary NASPC level (135 ± 88 μg g^{-1} creatinine) than non-smokers (76 ± 30 μg g^{-1} creatinine). A highly sensitive and selective LC/MS/MS isotope dilution method was successfully established. With an on-line clean-up system, this system is capable of routine high-throughput analysis and accurate quantitation of NASPC in urine. This could be a useful tool for health surveillance for acrylamide exposure in a population for future study. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: acrylamide; *N*-acetyl-*S*-(propionamide)cysteine; biomarker; liquid chromatography/tandem mass spectrometry

INTRODUCTION

Acrylamide (AA) is an important industrial chemical, whose polymers have various applications such as in water and soil treatment, tunnels and dam stabilization as a grouting agent.[1,2] AA is also present in tobacco smoke at levels of 1.1–2.3 μg per cigarette, probably owing to incomplete combustion or heating of organic matter.[3] Recently, AA has been detected in baked and fried starchy foods, largely derived from heat-induced reactions (Maillard reactions) between the free amino acid asparagines and the carbonyl group of reducing sugars, such as glucose, during baking and frying.[4–6] High AA contents have been reported in French fries, potato chips, tortilla chips, bread crust, crispbread and various baked goods and cereals.[6,7] Lately, daily dietary exposures to AA have been of great concern[5,8–11] because AA has been classified as a probable human carcinogen and neurotoxicant. It has been suggested that AA and its active metabolite glycidamide are alkylating agents. They contain an electrophilic group and could be attacked by protein nucleophilic sites and DNA bases to form protein and DNA adducts. These adducted bases may lead to mutations if not repaired prior to cell proliferation.[12,13]

To assess AA exposure, one of the adducted proteins, *N*-(2-carbamoylethyl)valine (CEVal), in hemoglobin has been used as a biomarker to differentiate long-term exposure to AA among smokers, laboratory workers and non-smokers.[12] AA can be detoxified using glutathione transferase through conjugation with glutathione, resulting in the urinary excretion of *N*-acetyl-*S*-(propionamide)cysteine (NASPC).[14,15] This metabolite accounts for 67 and 41% of the total metabolites present in rat and mouse urine, respectively.[14] Therefore, the urinary level of NASPC has

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been used as an alternative biomarker for CEVal to assess daily exposure to AA.[16]

NASPC analysis was first performed using high-performance liquid chromatography (HPLC) with fluorescence detection.[16] However, this method suffered from possible interference from the biomatrix and was time consuming when large numbers of samples were analyzed. This method requires derivatization to improve the sensitivity and avoid matrix effects. Alternatively, liquid chromatography/tandem mass spectrometry (LC/MS/MS) has high selectivity and sensitivity and no derivatization step is required. With the isotope dilution method, a targeted analyte could be quantitated reliably and accurately

In order to assess the daily human AA intake from a variety of foods, an isotope dilution LC/MS/MS method coupled with an on-line clean-up system was developed in this research to determine urinary NASPC.

EXPERIMENTAL

Chemicals

N-Acetyl-L-cysteine was purchased from ICN Biomedicals (Aurora, OH, USA). Acrylamide and $^{13}C_3$-labeled acrylamide were obtained from Sigma (St. Louis, MO, USA) and Cambridge Isotopes (Cambridge, MA, USA), respectively. Sodium hydroxide and ethyl acetate were purchased from Merck (Darmstadt, Germany). Hydrochloric acid and formic acid were supplied by Riedel-de Haën (Seelze, Germany). All analytical-grade solvents were provided by Tedia (Fairfield, OH, USA).

N-Acetyl-*S*-(propionamide)cysteine synthesis

NASPC was successfully synthesized in our laboratory from the reaction of *N*-acetyl-L-cysteine (2.4 g) with acrylamide (0.35 g) at 45 °C in 10 ml of acetone–water (1 : 1) (Fig. 1). This reaction was maintained under alkaline conditions (2.5 M) by adding 1 g of NaOH. After 5 h, the solution was neutralized with hydrochloric acid. NASPC was extracted with ethyl acetate (4 × 40 ml). The organic phase was dried using a rotary evaporator and the residue was dissolved in 95% acetonitrile. The product was purified and separated using an HPLC system consisting of a semi-preparative Betasil Diol-60 column (150 × 10 mm i.d., particle size 5 μm) (Thermo Electron, Waltham, MA, USA) and a UV detector set at 210 nm. The mobile phase was 95% acetonitrile delivered at a flow-rate of 3 ml min^{-1}. NAPSC eluted at 16 min and was collected and dried using a SpeedVac. NASPC ($^1CH_3{}^2CONH{}^3CH{}^4COOH{}^5CH_2S{}^6CH_2{}^7CH_2{}^8CONH_2$) was characterized by the ^{1}H and ^{13}C NMR chemical shifts using a Varian (Fort Collins, CO, USA) Mer-Vx-300 spectrometer. Deuterium oxide and methanol-d_4 were used as solvents. NMR chemical shifts are reported as δ values in parts per million (ppm). The splitting pattern abbreviations are as follows: s, singlet; br, broad; m, unresolved multiplet due to the field strength of the instrument. ^{1}H NMR (D_2O), δ 8.37 (br, 1H, NH), 4.36 (m, 1H, H-3), 3.04 (m, 1H, H-5a), 2.89 (m, 1H, H-5b), 2.86–2.78 (m, 2H, H-6), 2.55 (m, 2H, H-7), 2.03 (s, 3H, H-1). ^{13}C NMR (CD_3OD), δ 177.9 (C-8), 177.2 (C-4), 174.2 (C-2), 55.4 (C-3), 36.2 (C-7), 34.9 (C-5), 28.6 (C-6), 23.1 (C-1).

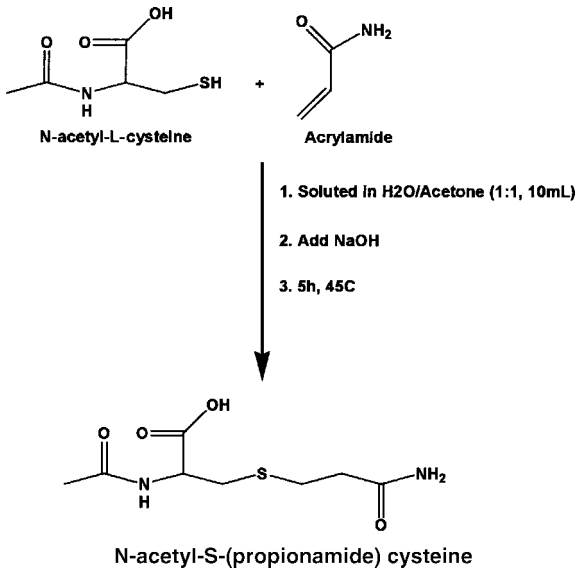

Figure 1. Synthesis of *N*-acetyl-*S*-(propionamide)cysteine (NASPC).

Automatic sample clean-up system

The column switching system consisted of a switching valve (Two-Position Microelectric Actuator, Valco, Houston, TX, USA) and a BETASIL DIOL cartridge (50 × 3 mm, i.d., particle size 5 μm) (Thermo Electron). The switching valve function was controlled using PE-SCIEX Analyst control software. The sample solution (20 μl) was loaded on to the cartridge using an autosampler (PE Series 200, Perkin-Elmer, Norfolk, CT, USA) and a quaternary pump (PE Series 200, Perkin-Elmer) delivered 20 mM ammonium acetate in 95% acetonitrile at a flow-rate of 1 ml min^{-1} as the loading and washing buffer. The cartridge was flushed with loading buffer for 5 min followed by valve switching to the injection position to inject the sample into the LC system. At 9 min, the valve was switched back to the loading position for equilibration of the cartridge and preparation for the next analysis.

High-performance liquid chromatography

After automatic sample clean-up, the sample was automatically delivered into a microbore BETASIL DIOL column (150 × 2.1 mm i.d., particle size 5 μm) (Thermo Electron). The gradient mode was used to achieve the desired sample separation using mobile phase A (95% acetonitrile containing 0.1% formic acid) and mobile phase B (5% acetonitrile containing 0.1% formic acid) delivered at a flow-rate of 600 μl min^{-1}. Mobile phase A (100%) was used from 0 to 5.5 min followed by a fast gradient applied to switch to 40% mobile phase B within 0.5 min. Mobile phase B (40%) was maintained for 4 min before a quick ramp to 100% mobile phase B, which was maintained for 1.5 min, then back to 100% mobile phase A, which was maintained for another 15 min.

Electrospray ionization tandem mass spectrometry (ESI-MS/MS)

An API 3000 triple-quadruple mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, ON, Canada) equipped

with a TurboIonSpray source was used. NASPC standard solution (5 mg l^{-1}) was infused into the mass spectrometer at 10 µl min^{-1} to optimize the LC/MS/MS operating parameters. A Q1 full-scan NASPC spectrum was conducted first to obtain $[M + H]^+$ and $[M - H]^-$ ions using the positive and negative modes, respectively. The $[M + H]^+$ and $[M - H]^-$ ions were selected to acquire the product ion scans for selecting the most distinctive precursor/product NASPC ion pairs. The mass spectrometer was operated in the negative multiple reaction monitoring (MRM) mode for NASPC quantitation. This included m/z 233.0 → 104.0 for NASPC with the dwell time set at 150 ms and m/z 236.0 → 107.0 for $[^3C_{13}]$NASPC with the dwell time set at 100 ms.

The spraying needle voltage was set to −3800 V for the negative mode. The nebulizer and curtain gas flow-rates were set at 12. Collision-activated dissociation (CAD) and turbo gas flow-rate were set at 4 and 8, respectively. The source heater probe temperature was set at 450 °C. Data acquisition and quantitative processing were accomplished using Analyst software, Version 1.1 (Applied Biosystems).

Urine samples

Human urine samples were collected from 70 apparently health male military officers (33 non-smokers and 37 smokers). The smoking status of these subjects was obtained using a questionnaire. The subjects' ages ranged from 20 to 46 years with body mass index 17.30–28.40. Military officers were chosen owing to their similar diet and life style. Urinary NASPC concentrations were adjusted with creatinine and expressed in µg g^{-1} creatinine.

Each urine sample (200 µl) was mixed with 700 µl of 95% acetonitrile containing 0.1% formic acid and 100 µl of internal standard ($[^{13}C_3]$NASPC, 100 µg l^{-1}). After centrifugation at 10 000 r.p.m. for 3 min, sample solution (20 µl) was injected into the analytical system. No further sample preparation procedures were needed. The urinary creatinine was determined as the creatinine–picrate complex by spectrophotometry (U-2000, Hitachi, Tokyo, Japan) using a wavelength of 520nm in Kaohsiung Medical Hospital.[17]

Statistical methods

Data were analyzed using a statistical package (SPSS, Statistical Package for the Social Sciences). Statistically significant differences were determined using p-values obtained with Student's t-test.

RESULTS AND DISCUSSION

Characterization of *N*-acetyl-*S*-(propionamide)cysteine using mass spectrometry

After NASPC and $[^{13}C_3]$NASPC had been synthesized and purified, the chemical structure of NASPC was determined and confirmed by NMR, as described in the Experimental section. NASPC and $[^{13}C_3]$NASPC were further characterized using electrospray ionization tandem mass spectrometry (ESI-MS/MS). Full-scan spectra were obtained by operating the ESI-MS/MS system in the positive and negative modes (Fig. 2) In positive mode, the molecular ion is the protonated NASPC ($[M + H]^+$, m/z 235), and the ion at m/z 257

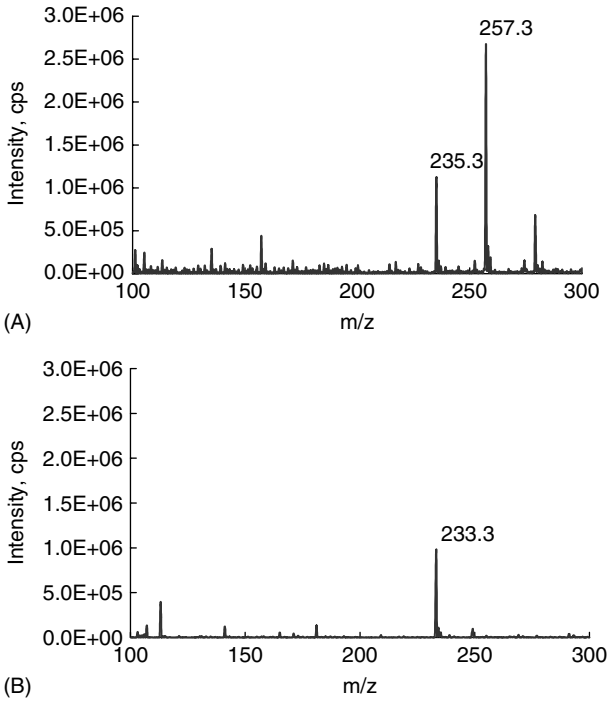

Figure 2. (A) Positive and (B) negative full-scan mass spectra of NASPC obtained by infusion of 5 µg ml^{-1} standard solution into the MS system.

is sodium-adducted NASPC $[M + Na]^+$ (Fig. 2(A)). In the negative mode, the predominant ion is m/z 233, corresponding to $[M - H]^-$ (Fig. 2(B)). The abundance of the $[M + H]^+$ ion appears approximately equal to that of $[M - H]^-$ in the full-scan mode. Product ion scan spectra were obtained by selecting positive and negative precursor ions to collide with nitrogen. The fragment ion abundance at m/z 104 operated in the negative mode was three times higher than that in the positive mode (Fig. 3). The negative mode will be more sensitive than the positive mode for analysis of NASPC if m/z 104 is monitored. Similar mass spectra were obtained for $[^{13}C_3]$NASPC, except that the corresponding ions were 3 Da greater than those of unlabeled NASPC (data not shown). The negative MRM mode was applied to monitor product ions at m/z 104 and 107 for NASPC and $[^{13}C_3]$NASPC fragmented from precursor ions at m/z 233 and 236, respectively (Fig. 4). The mass spectrometer was tuned by direct infusion of 5 mg l^{-1} NASPC standard solution at a flow-rate of 10 µl min^{-1} to optimize the ion path voltages.

Precision and accuracy of the isotope dilution LC/MS/MS method

A linear calibration curve from 10 to 500 µg l^{-1} was obtained using known amounts of standards in a blank urine matrix; each calibration solution contained 100 µl of 100 µg l^{-1} $[^{13}C_3]$NASPC and yielded an equation for the line of $y = 0.0015x - 0.0087$ ($r^2 = 0.9997$), as shown in Fig. 5. The detection limit was estimated to be 5 µg l^{-1} (0.4 pmol) on-column (signal-to-noise ratio = 5). The mean recovery was calculated as the analyte concentration in the urine and loading buffer (working solution) divided by analyte concentration in the loading buffer. A working solution was prepared in a mixture of urine and loading buffer (95%

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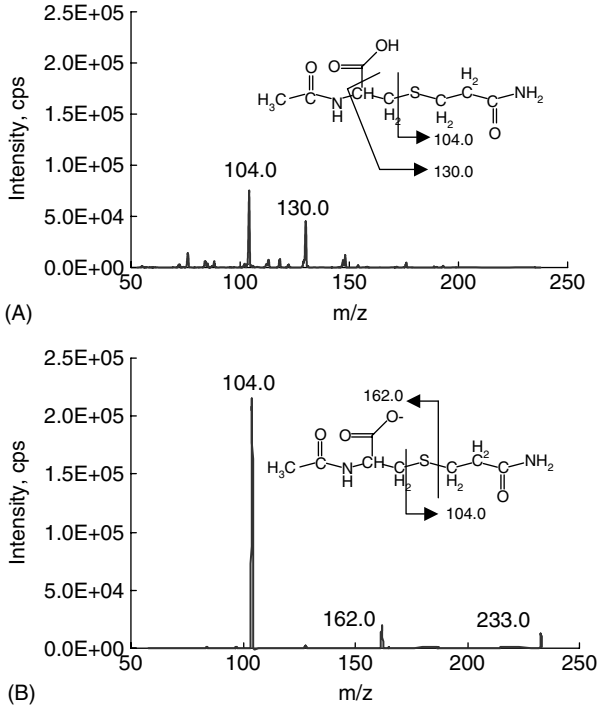

Figure 3. (A) Positive and (B) negative product ion scan mass spectra of NASPC obtained by infusion of 5 μg ml^{-1} standard solution into the MS system.

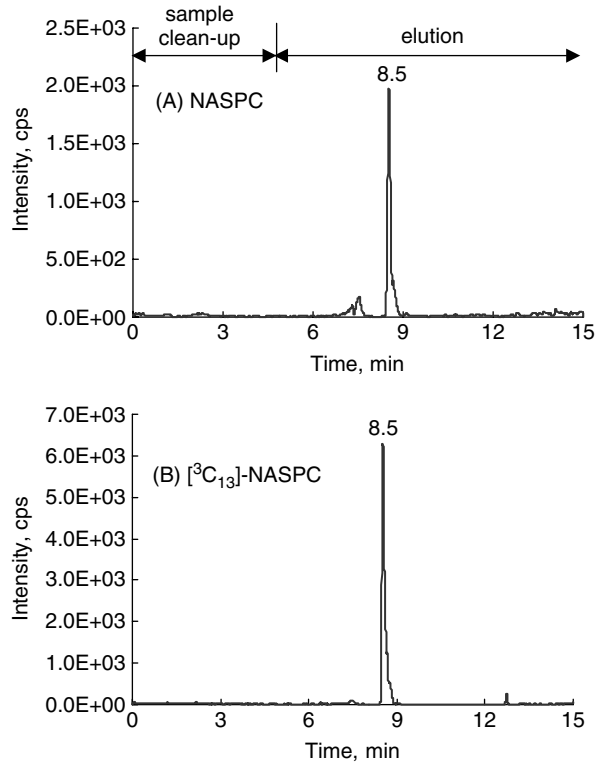

Figure 4. Chromatograms of (A) NASPC and (B) [$^{3}C_{13}$]NASPC generated from analysis of a urine sample collected from a non-smoker by using the on-line clean-up LC/MS/MS method.

acetonitrile containing 0.1% formic acid) at a concentration of 50 μg l^{-1} and was measured using the on-line clean-up device followed by LC/MS/MS analysis. The mean recovery

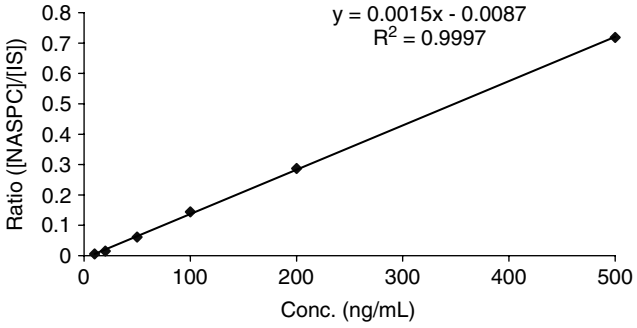

Figure 5. Calibration curve for quantification of NASPC in human urine.

($n = 6$) was found to be 86%.

Relative standard deviation (RSD) is used to express the precision. It was calculated by dividing the standard deviation by the mean of the data set. Accuracy is expressed as the percentage relative error (RE), which is given by the expression: $[(x_i - x_t)/x_t] \times 100\%$, where x_i is the measured value and x_t is the true value. As for the intra-day variation, the sample was also prepared in the mixture of urine and loading buffer at 50 μg l^{-1} and was analyzed three times, morning, afternoon and evening, within one day. Two analyses were conducted at each time point to give a total of six analyses. Based on the data generated from six replicate 50 μg l^{-1} NASPC spiked urine analyses, the precision and accuracy of the method were calculated as 13% and 2%, respectively.

Wu *et al.*[16] established an HPLC/fluorescence detection method for measuring urinary NASPC. The detection limit and recovery were reported to be 1 pmol and 94%, respectively. In comparison with that study, our method provided a better detection limit. The HPLC/fluorescence method requires sample purification and derivatization with *o*-phthalaldehyde and a total time of 4 h was required for hydrolysis and derivatization. Such a time-consuming procedure could limit its application to the analysis of human samples. With our on-line clean-up system coupled with LC/MS/MS for NASPC detection in human urine, the total analysis time is only 12 min without any further hydrolysis and derivatization.

Application to human urine samples

Our method was applied first to investigate the effect of cigarette smoking on the urinary level of NASPC. Urine samples were collected from 70 apparently healthy military officers. The mean urinary levels of NASPC observed for smokers and non-smokers are plotted in Fig. 6. The mean urinary level of NASPC for the non-smoker group was 76 ± 30 μg g^{-1} creatinine and for the smoker group 135 ± 88 μg g^{-1} creatinine. Smokers had a significantly higher urinary level of NASPC than nonsmokers ($p < 0.05$). If we did not use creatinine to adjust the urine samples, the NASPC concentrations in the urine were 113 ± 22 and 159 ± 43 μg l^{-1} in the non-smoker and smoker groups, respectively. It has been reported that AA is widely present in cigarettes.[3] To our knowledge, our study might be the first study to show that AA exposure through cigarette smoking results in an increase in urinary NASPC.

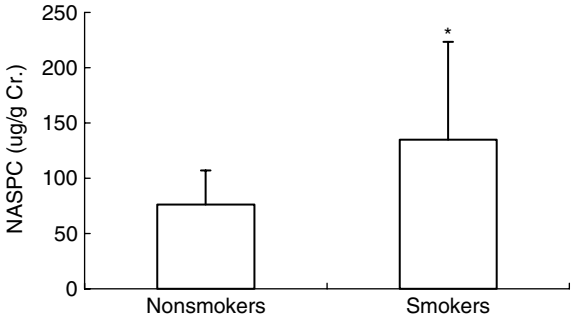

Figure 6. NASPC contents (mean ± SD) in urine samples from non-smokers and smokers. $^{*}p < 0.05$.

A previous study collected 24 h urine samples from 41 AA-exposed workers at an AA factory and measured the NASPC urinary levels.[16] It was found that the NASPC urinary concentrations ranged from 1872 to 74 412 $\mu g\ l^{-1}$. The urinary levels of NASPC for both the smoker and non-smoker groups in our study were at least 10 times lower than those for the exposed workers at the AA factory. One possible reason could be that the workers at the AA factory experienced relatively high-level exposure to AA, resulting in a high level of urinary NASPC. Another possibility might be that the HPLC/fluorescence detection method might have overestimated the NASPC levels in urine, especially as crude urine samples contain considerable amounts of cross-reacting substances and other structurally related compounds. It has been suggested that the HPLC/fluorescence method has low specificity.[18]

CONCLUSIONS

A highly specific and sensitive isotope dilution LC/MS/MS method for the determination of urinary NASPC was successfully established. Using an on-line clean-up system, this method is capable of routine and high-throughput analysis for monitoring AA exposure. The isotope dilution method also provides a high degree of confidence in the method's performance. We believe that the proposed method could be a good tool for assessing AA exposure in future studies, particularly for assessing low exposures from daily intakes of high-temperature processed foods.

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