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Quantitative analysis of heterocyclic amines in urine by liquid chromatography coupled with tandem mass spectrometry

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ABSTRACT

A sensitive, reproducible, and rapid analytical method for the analysis of trace-level heterocyclic amines (HCAs) that are expected to have high levels of human exposure was developed. Liquid–liquid extraction (LLE) with dichloromethane (DCM) followed by solid-phase extraction (SPE) was carried out. Liquid extraction with DCM under basic conditions was efficient in extracting HCAs from urine samples. For further purification, mixed mode cationic exchange (MCX) cartridges were applied to eliminate the remaining interferences after liquid extraction. Separation and quantification were performed by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) in selected reaction monitoring (SRM) mode. The overall recoveries ranged between 71.0% and 113.6% with relative standard deviations (RSDs) of 5.1% to 14.7% for the entire procedure. The limits of detection (LODs) and limits of quantification (LOQs) of the proposed analytical method were in the ranges of 0.04 to 0.10 ng/ml and 0.15 to 0.36 ng/ml, respectively. This method was applied to the analysis of monitoring in urine samples for Korean school children, and the results demonstrated that the method can be used for the trace determination of HCAs in urine samples.

Assessment of human exposure to heterocyclic amines (HCAs)[1] has been of increasing concern since the discovery of mutagenic activity in smoke particles from broiled fish [1]. These compounds are formed in thermally treated protein-rich food at the nanogram/gram (ng/g) level [2] but are also detected in several other environments, including cooking fumes, cigarette smoke, air, rain, and river water [3]. So far, the toxicity of HCAs related to carcinogenic and mutagenic response has been evaluated in experimental animal and cell models [4,5]. For an estimation of the overall risk to people, epidemiological studies depending on dietary habit, race, age, and gender should be investigated extensively [6,7]. However, the lack of information for quantitative data on HCA exposure limits the ability to evaluate the health risk and safety management. To integrate the data of human exposure, urine has been used as a biological fluid that has the advantage of simple and noninvasive collection. In spite of the useful matrix for monitoring, the parent HCAs of urinary excretion were detected at sub-nanogram levels because HCAs are rapidly metabolized by two different pathways: detoxification and activation [8,9]. During the metabolic process, hydroxylation, sulfation, glucuronidation, and adduct form with macromolecules such as protein and DNA to show that carcinogenic effects may also be involved [10–17]. Therefore, a highly sensitive, selective, and reliable method for the determination of HCAs is required due to their low concentrations and the high complexity of the matrix.

Previously, we reported that a multiple solid-phase extraction (SPE) method was used with liquid chromatography coupled with mass spectrometry (LC–MS) for the analysis of HCAs in human urine [18]. However, there are several drawbacks to determine HCAs in urine samples using the previous method with respect to achieving large-scale analysis for biomonitoring. Here, we developed a more sensitive, reproducible, and rapid method for the analysis of eight HCAs for biomonitoring study. The target analytes, which are expected to have high levels of exposure through the meat and dairy products of South Korea, are shown along with the precursor for the formation in Fig. 1. We employed simple liquid–liquid extraction (LLE), followed by one step (SPE) for the sample preparation and the selected reaction monitoring (SRM) technique with tandem mass spectrometry (MS/MS), for the quantification of HCAs. The method was validated with respect to precision, accuracy, linearity,

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[1] *Abbreviations used:* HCA, heterocyclic amine; SPE, solid-phase extraction; LC–MS, liquid chromatography coupled with mass spectrometry; LLE, liquid–liquid extraction; SRM, selected reaction monitoring; MS/MS, tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; IS, internal standard; MeOH, methanol; DCM, dichloromethane; HPLC, high-performance liquid chromatography; MCX, mixed mode cationic exchange; QC, quality control; QA, quality assurance; MS/MS, tandem mass spectrometry; CE, collision energy; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation; UV, ultraviolet; ESI, electrospray ionization.

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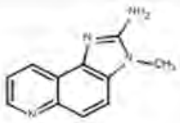
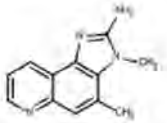
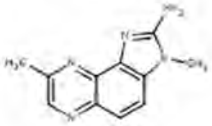
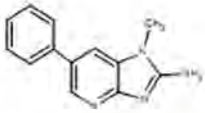
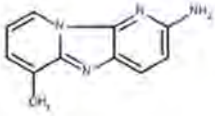
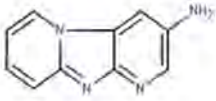
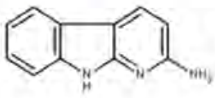
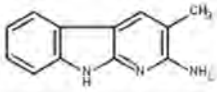
Chemical name	Abbreviation	Structure	Molecular mass and properties	Precursor
Amino-imidazo-azzarene 2-amino-3-methylimidazo [4,5-f]quinoline	IQ		198.2239 pKa=3.5	Creatine, Gly, Phe, Ser, Pro, Fructose, Glucose
2-amino-3,4-dimethylimidazo [4,5-f]quinoline	MeIQ		212.2505	Creatine, Ala, Fructose
2-amino-3,8-dimethylimidazo [4,5-f]quinoxaline	MeIQx		213.2385	Creatine, Gly, Ala, Thr, Lys, Fructose, Glucose, Ribose
2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine	PhIP		224.2612 pKa=5.6	Creatine, Phe, Glucose
Amino-carboline 2-amino-6-methyldipyrido [1,2-a:3′,2′-d]imidazole	Glu-P-1		198.2239 pKa=6.0	Glutamic acid
2-amino-dipyrodo[1,2-a:3′,2′-d]imidazole	Glu-P-2		184.1973 pKa=5.9	Glutamic acid Casein
2-amino-9H-pyrodo[2,3-b]indole	AαC		183.2093 pKa=4.4	Soybean Globulin
2-amino-3-methyl-9H-pyrodo[2,3-b]indole	MeAαC		197.2358	Soybean Globulin

Fig.1. Abbreviations, structures, and properties of target HCAs.

limit of detection (LOD), and limit of quantification (LOQ). The proposed method was applied successfully to the analysis of monitoring in urine samples for Korean school children and can offer the assessment of human health risk of HCA exposure.

Materials and methods

Reagents and chemicals

Neat standards of eight HCAs (IQ, Glu-P-1, Glu-P-2, MeIQx, MeIQ, PhIP, AαC, and MeAαC; see Fig. 1 for full chemical names) were purchased from Toronto Research Chemicals (Toronto, Canada). 1-Naphthyl amine as the internal standard (IS) and β-glucuronidase were purchased from Sigma (St. Louis, MO, USA). Methanol (MeOH), Acetonitrile, dichloromethane (DCM), ammonium acetate, and acetic acid were of high-performance liquid chromatography (HPLC) grade and were purchased from Fisher Scientific (Pittsburgh, PA, USA). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). An Oasis MCX (mixed mode cationic exchange) cartridge (30 mg, 1 ml) was obtained from Waters (Milford, MA, USA). Ammonia solution (25%) and hydrochloric acid (37% fuming) were obtained from Merck (Darmstadt, Germany).

Samples

A 500-ml pooled urine sample that did not contain the eight HCAs was prepared and used in spiking experiments for quality control (QC) and quality assurance (QA). For the stability test, an individual 2-ml aliquot of a pooled urine sample was spiked with 5 ng of HCAs and then stored at −18 °C until batch analysis of samples. All urine samples from Korean school children were provided by the Korea Food and Drug Administration (KFDA).

Sample preparation

All samples were thawed and centrifuged before analysis. For the enzymatic deglucuronidation and hydrolysis, 50 μl of β-glucu-

ronidase was added to 2 ml of urine and incubated at 37 °C for 30 min. Then, acid hydrolysis was carried out, adding 350 µl of hydrochloric acid (1 M) and heating at 90 °C for 1 h. After hydrolysis, 200 µl of sodium hydroxide was added and extracted three times with 2 ml of DCM. The extract was loaded onto an MCX cartridge preconditioned with 1 ml of DCM. After washing with 1 ml of 0.1 M HCl and 1 ml of MeOH, the eluent was collected using 5 ml of 10% NH_3 in MeOH. The eluent was dried in a SpeedVac concentrator (Savant, Farmingdale, NY, USA) and reconstituted with 100 µl of 1-naphthylamine (1 µg/ml in MeOH) as an IS. Finally, 5 µl of sample was injected onto the LC–MS/MS (tandem mass spectrometry) system for analysis. Fig. 2 shows the analytical procedure for the determination of HCAs in urine.

LC–MS/MS analysis

The chromatographic separation of extracts was performed using a Finnigan Surveyor Plus HPLC system (Thermo Finnigan, San Jose, CA, USA) equipped with an Ascentis Express RP-Amide column (150 × 2.1 mm, 2.7 µm, particle size). The analytical column was protected by a Phenomenex C18 guard column (4 × 2.1 mm, 2 µm, particle size). The mobile phase was a binary mixture of 10 mM ammonium acetate in water and acetonitrile adjusted to pH 5.0 with acetic acid in a gradient elution mode at a flow rate of 150 µl/min. Gradient conditions were as follows: from 10% acetonitrile and 90% ammonium acetate (10 mM at pH 5.0) to 25% acetonitrile and 75% ammonium acetate for 4 min, then to 30% acetonitrile and 70% ammonium acetate for 6 min, then from this composition to 70% acetonitrile and 30% ammonium acetate for 10 min, and then changed to the initial mobile phase composition for 10 min. A 5-µl sample aliquot was injected into the HPLC. Solvents were degassed online, and the column temperature was maintained at 30 °C. Mass spectrometric experiments were performed using a Thermo Finnigan TSQ Quantum Ultra EMR triple quadrupole mass spectrometer. The samples were analyzed in positive ion electrospray ionization with a spray voltage of 4 kV under an N_2 sheath gas flow rate of 30 arbitrary units. The capillary temperature was maintained at 275 °C. In the SRM mode for the quantification, the product ion of each parent compound was monitored at the individual collision energy (CE). Optimization of the CE for each individual compound was done by infusion of the compound directly into the LC effluent using a syringe pump. Data acquisition was performed with Xcalibur version 2.0.7 software, and peak integration and calibration were obtained with Qual Browser version 2.0.7 software.

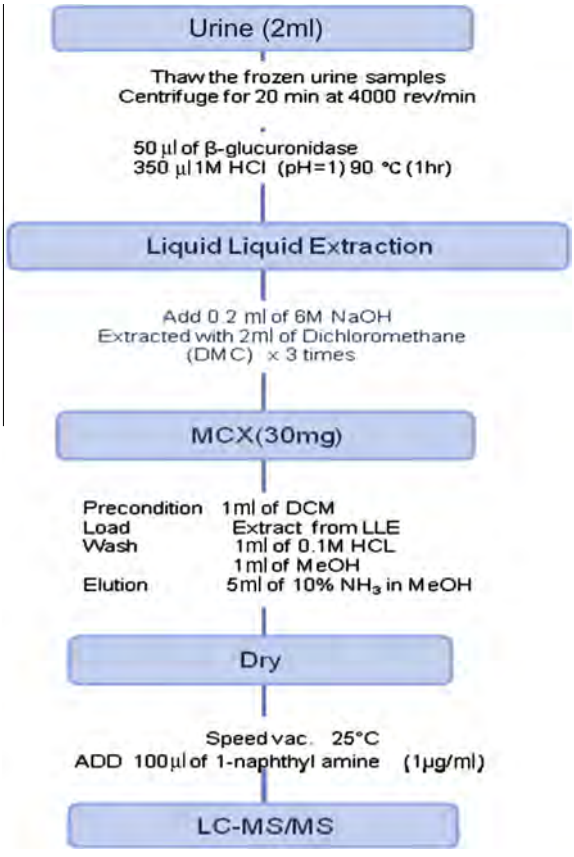


Fig.2. Analytical procedure for the determination of HCAs in urine samples.

Method validation

The method was validated for linearity of calibration, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) in accordance with our previous study. Linearity was assessed by the analysis of standard solutions in the range of 0.025 to 5 ng/ml. For the recovery measurement, 2 ml of blank urine samples spiked with the known amount of the analytes (the concentrations of 0.5 and 1.5 ng/ml in urine sample) were prepared and determined by the analytical procedure as shown in Fig. 2. The accuracy and precision were assessed by recovery experiments using triplicate urine samples spiked with two different concentrations compared with the pure authentic standards. LOD and LOQ were investigated by analyzing nine individual blank urine samples spiked with 0.5 ng of standard mixture for HCAs. Stability studies were performed for a period of 8 months at a concentration of 2.5 ng/ml to ensure the reliability of results related to sample handling and storage of urine.

Results and discussion

Optimization of extraction and cleanup conditions

The previous method using three multiple SPE cartridges needed because of sophisticated manipulation leads to poor recovery and reproducibility. Occasionally, the malfunction of blockages of cartridges occurred during the sample introduction to SPE cartridges because of viscosity of urine. Furthermore, the manual approach involving multistep elution onto multiple cartridges of SPE is not convenient, and the achievement of good repeatability and reproducibility is provided under tightly controlled conditions. Instead of the multistep procedures, LLE was considered before a single step of SPE. DCM and ethyl acetate as an extraction solvent have been commonly used [19,20]. DCM, which is denser than water, was applied in this study because it allows the easy separation of water and the conducting SPE step. When 10 ng of HCAs was spiked into 3 ml of water, the overall recoveries of the amino-imidazo-azzarene group that has polar compounds (IQ, MeIQ, MeIQx, and PhIP) ranged between 30.7% and 40.4%, whereas those of the amino-carboline group that has nonpolar compounds (Glu-P-1, Glu-P-2, AαC, and MeAαC) ranged between 50.2% and 74.7%, using only DCM as an extraction solvent. However, the recoveries of both groups increased significantly with extraction under basic conditions after the addition of 200 µl of 6 M NaOH into water. The relative standard deviations (RSDs) were less than 6.1%, and recoveries were in the range of 70.6% to 98.4% under basic conditions from the spiking experiment performed in triplicates. These values are considered as the acceptable range for percentage recovery and RSD; thus, this extraction method was combined with the further SPE procedure. As an SPE cartridge, the Oasis MCX, which is a mixed mode cation exchange sorbent to achieve higher selectivity and sensitivity for extracting basic compounds, was selected for

further cleanup based on our previous study. In contrast to the previous study, the final elution volume on the Oasis MCX cartridge by DCM increased from 3 to 5 ml of 10% NH_3 in MeOH to obtain higher recoveries of MeAαC, AαC, and PhIP. These compounds were strongly retained on the Oasis MCX cartridge. Then, an additional 2 ml of eluent was needed to recover all HCAs, as shown in Fig. 3. The overall recoveries during the SPE step were in the range of 76.9% to 106.6%, with RSDs less than 14.4% in triplicates. Fig. 4 shows the ultraviolet (UV) chromatograms with detection at 213 nm of spiked urine samples after each extraction and cleanup step. The matrix effect of the urine sample was effectively eliminated by LLE under basic conditions combined with Oasis MCX cartridge. In the fraction of DCM after LLE, target HCAs were clearly observed in the chromatogram from matrix. After consecutive LLE and SPE, the baseline noise of the chromatogram was reduced to increase the sensitivity of HCAs.

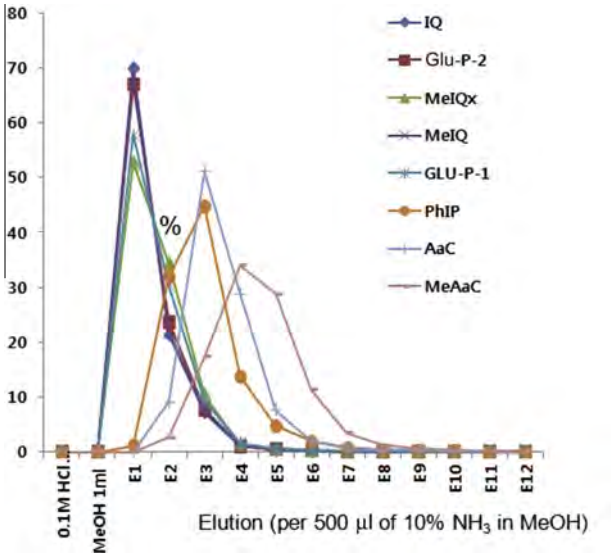


Fig.3. Elution profile of HCAs from Oasis MCX cartridge.

Optimization of LC–MS/MS condition

Regarding the separation of HCAs, poor peak shapes and inadequate retention on the classic C18 and C8 reversed-phase columns were observed in our previous study. The less polar compounds PhIP, AαC, and MeAαC are strongly retained on these reversed-phase columns, consequently resulting in long analysis time [21,22]. Alternatively, the RP-Amide column embedded polar group stationary phase was used to achieve the appropriate separation performance for both polar and less polar amines. This amide phase reduces silanol interactions with basic analytes, improving peak shape with shorter analysis time than C18 and C8 columns. The buffer composition, pH, and gradient elution program were established to achieve the best chromatographic separation on the amide column, as shown in Fig. 5.The mass spectra of HCAs showed predominant protonated molecular ions without fragmentations under the electrospray ionization (ESI)(+)–MS condition. For proper identification of HCAs in urine, the MS/MS experiment in SRM mode was performed. The methyl-loss fragment ion, $[M+H-CH_3]^+$, was produced as the base peak in the MS/MS spectrum for the amino-imidazo-azzarene group. On the other hand, the ammonia-loss ion, $[M+H-NH_3]^+$, was the predominant fragment for the other amino-carboline group. Each individual full-scan product ion spectrum at different CEs is represented as shown in Fig. 5.

Method performance

To determine the sensitivity of the current LC–SRM method using a triple quadrupole MS analyzer, the standard mixture

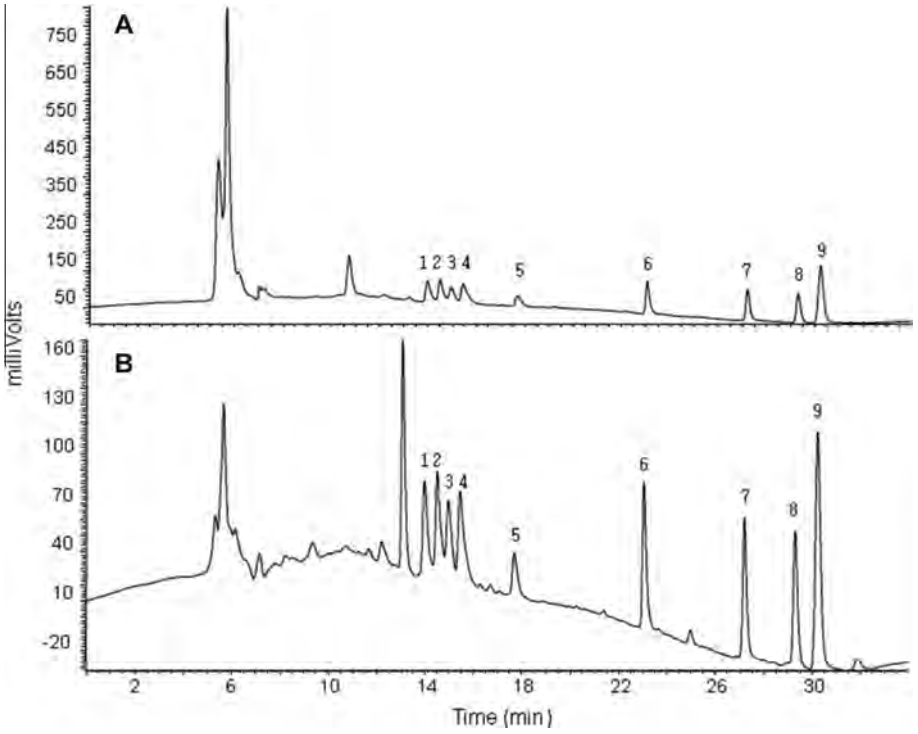


Fig.4. UV chromatograms of urine spiked with 50 ng/ml HCAs purified by LLE (A) and LLE and MCX SPE cartridge (B). Peak identities are as follows: (1) IQ; (2) Glu-P-2; (3) MeIQx; (4) MeIQ; (5) Glu-P-1; (6) PhIP; (7) AαC; (8) MeAαC; (9) IS.

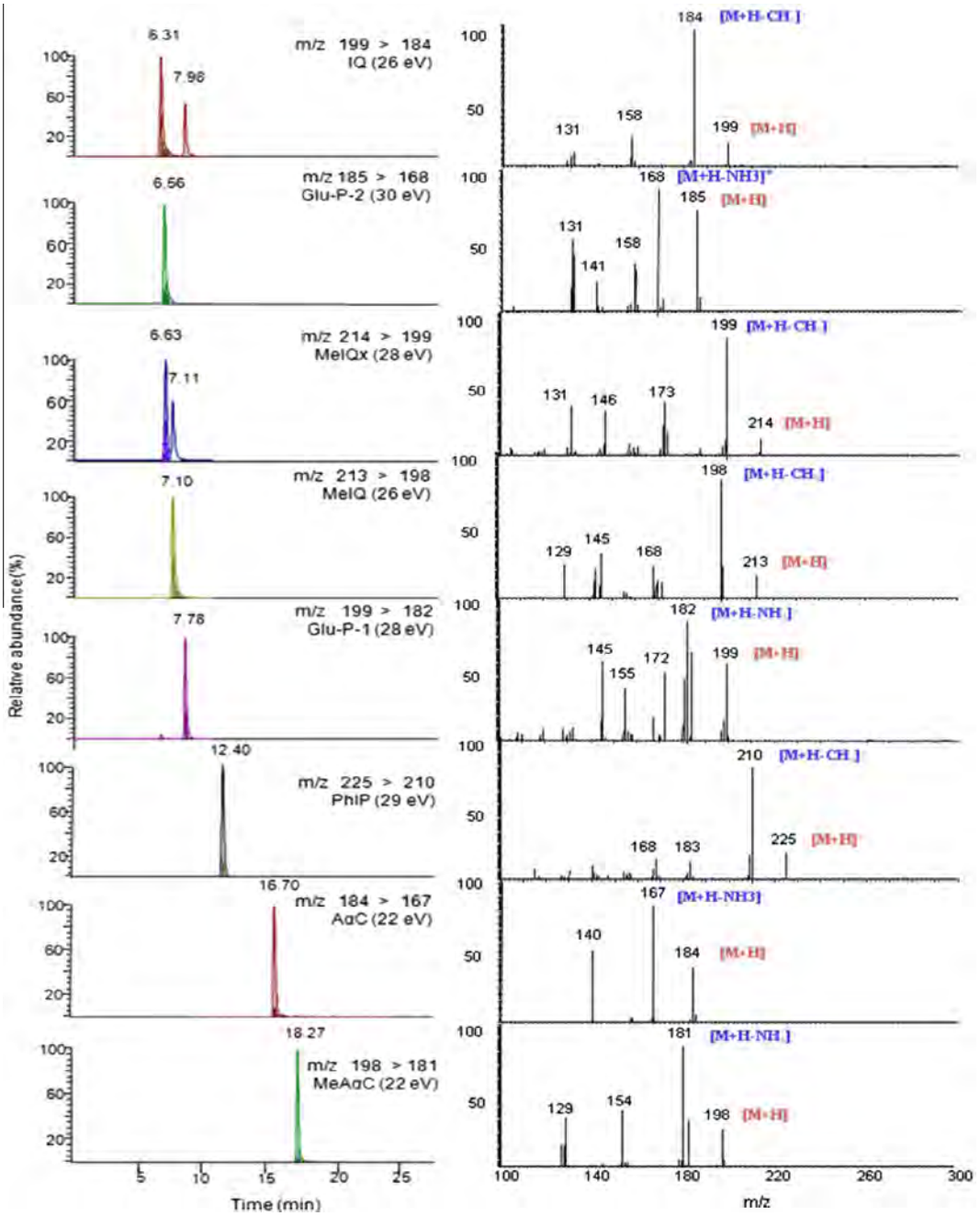


Fig.5. LC–ESI(+) MS/MS chromatogram of a standard solution with eight heterocyclic amines (5 ng/ml). Each individual full-scan product ion spectrum at different collision energies is represented on the right.

Table 1
Calibration equations, detection limits (LOD and LOQ), and recoveries of HCAs in urine samples.

Compound	Regression line			LOD (ng/ml)	LOQ (ng/ml)	Concentration			
	Slope	Intercept	Correlation coefficient			0.5 ng/ml		1.5 ng/ml	
						Accuracy (%)	Precision (%RSD)	Accuracy (%)	Precision (%RSD)
IQ	7.1990	0.1261	0.9993	0.04	0.15	92.1	10.4	78.5	10.6
Glu-P-1	1.6227	−0.0433	0.9996	0.05	0.18	71.0	14.7	74.0	10.8
Glu-P-2	1.4810	0.0428	0.9992	0.06	0.22	74.0	11.5	76.1	12.2
MeIQx	3.2861	0.0395	0.9998	0.05	0.17	86.6	10.4	78.4	6.8
MeIQ	9.1088	0.1577	0.9997	0.05	0.16	100.6	7.7	93.1	13.8
PhIP	3.5502	0.0234	0.9993	0.07	0.23	113.6	5.1	93.6	7.2
AαC	1.4391	0.0356	0.9990	0.07	0.23	102.8	7.3	95.1	9.8
MeAαC	1.3029	0.0171	0.9998	0.10	0.36	103.4	7.0	99.0	10.5

solution of eight HCAs was diluted and injected into the LC–MS/MS system. Instrumental detection limits (signal-to-noise ratio of 3) of HCAs ranged from 0.1 to 0.5 pg; these values were 20 to 200 times lower than those in the previous ion trap mass analyzer. The use of ion trap offered higher scan speed and provided very useful analyte mass spectra for the unambiguous identification of compounds. Nevertheless, the sensitivity obtained with the ion trap was not as good as that obtained with the triple quadrupole MS analyzer in SRM mode. Thus, lower sample consumption of 2 ml instead of 3 ml of urine and a 10 times lower calibration limit were able to be adopted in this study.

Seven levels of standard mixtures of HCAs (0.025, 0.05, 0.25, 0.5, 1.0, 2.5, and 5.0 ng/ml) that cover the range of concentrations expected of the samples were used to define the linearity. The linear

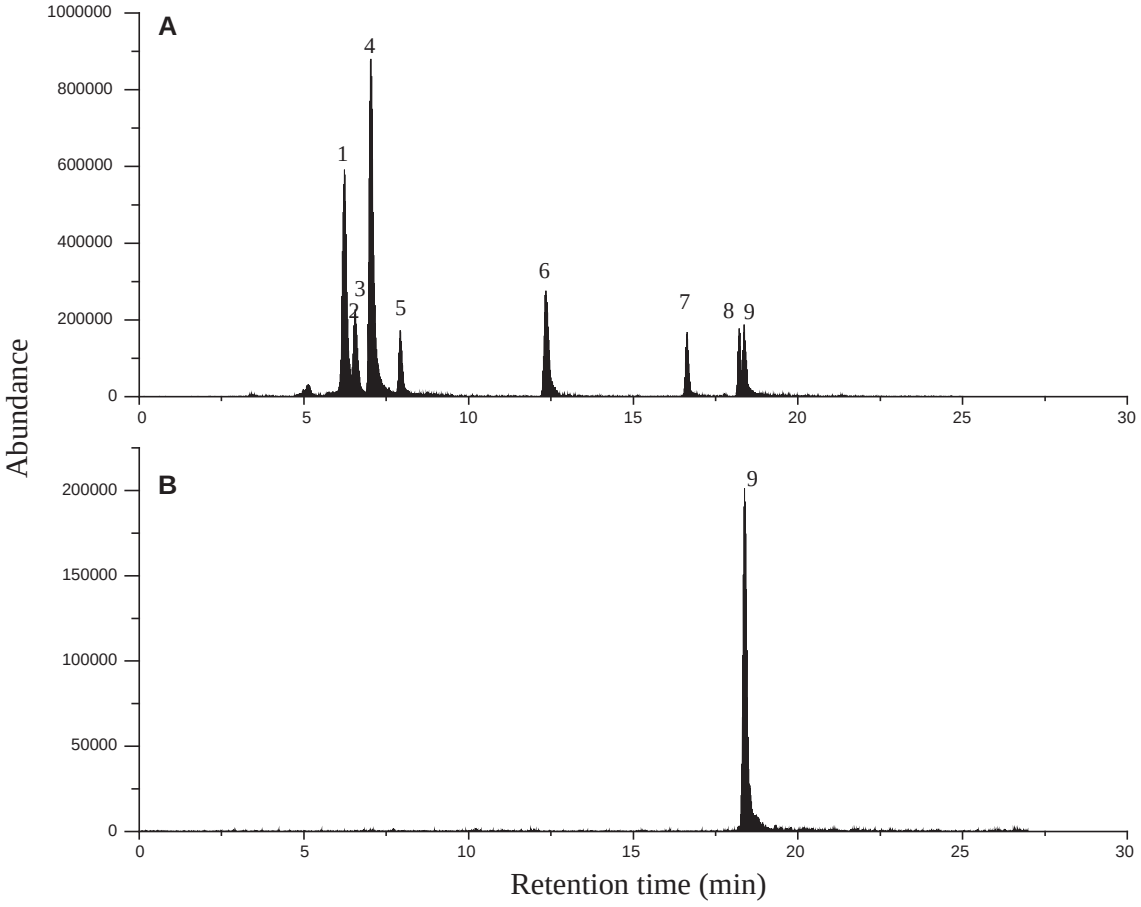


Fig.6. SRM chromatograms of a urine sample spike at LOQ level (A) and urine blank (B). Peak identities are as follows: (1) IQ; (2) Glu-P-2; (3) MeIQx; (4) MeIQ; (5) Glu-P-1; (6) PhIP; (7) AαC; (8) MeAαC; (9) IS.

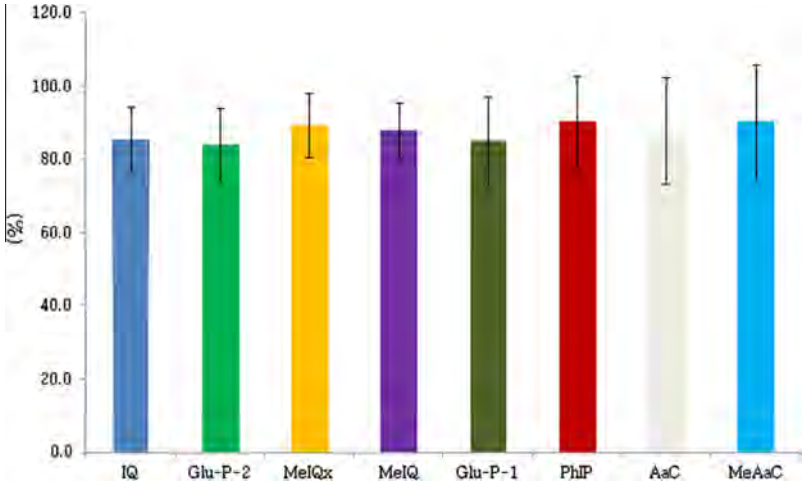


Fig. 7. Stability of heterocyclic amines in urine samples stored for 8 months.

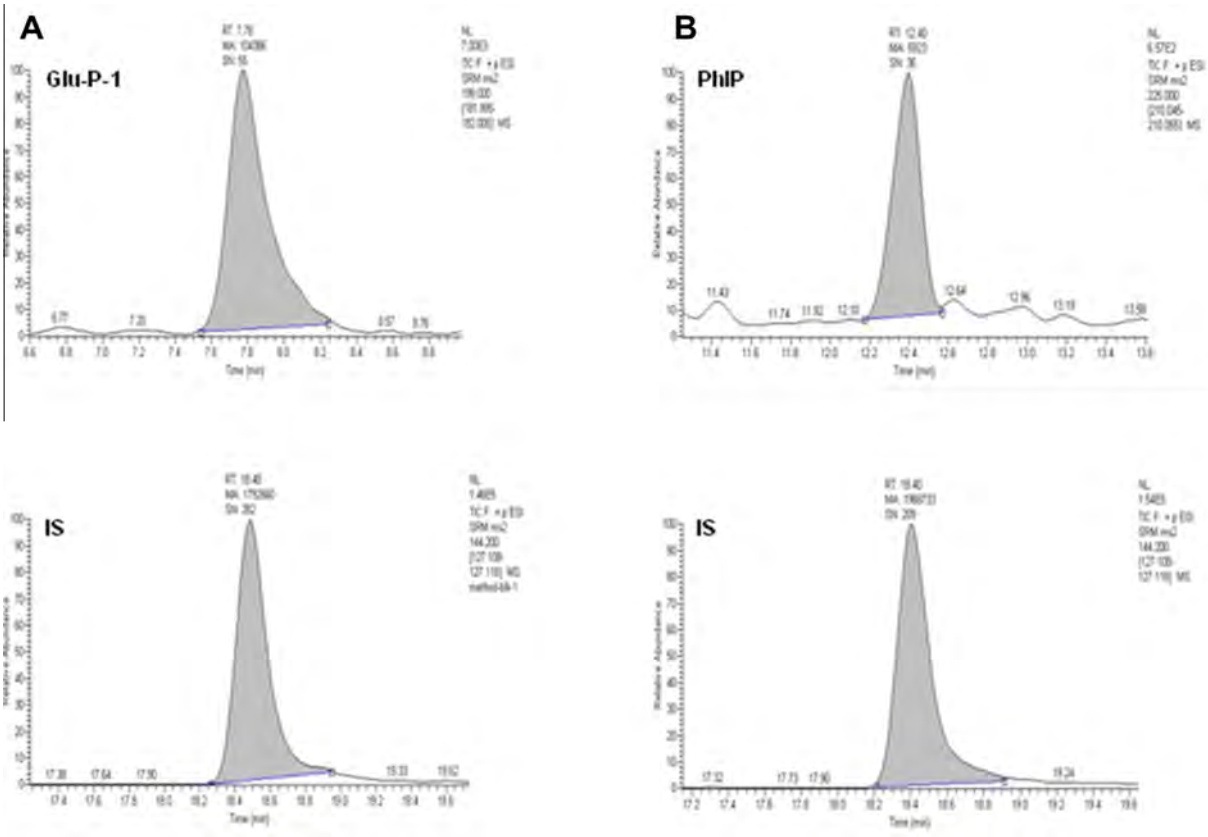


Fig. 8. SRM chromatograms of Glu-P-1 (A) and PhIP (B) with internal standard in a positive urine sample.

equations were obtained by the peak ratios for HCAs against the internal standard of 1-naphthylamine. Table 1 shows linear regression equations, correlation coefficients and linear ranges obtained in the analysis of eight HCAs. The correlation coefficients were better than 0.9990 to provide satisfactory results in quantitative analysis. To validate the accuracy and precision of the analytical method, three replicate analyses of pooled urine samples spiked with HCAs for the two levels (0.5 and 1.5 ng/ml) were performed. The percentage recovery was calculated by the response of extracted urine by the result of unextracted standards that represent 100% recovery. The accuracy and precision were assessed by the average recovery and percentage RSD of three results at each concentration, as shown in Table 1. The average recovery of HCAs ranged from 71.0% to 113.6%, with RSDs ranging from 5.1% to 14.7%. LOD and LOQ were determined by performing nine analyses with 2 ml of urine spiked with 0.5 ng of the HCA mixture and were defined according to the previous study. These values (0.04–0.1 ng/ml for LOD) were several orders of magnitude lower than previous results (0.25–0.48 ng/ml for LOD). SRM chromatograms of a urine sample spiked at the range of the LOQ level and a urine blank are provided, as shown in Fig. 6. There was no presence of a quantifiable analyte signal in the extract of blank matrix.

Stability studies were performed on QA samples to evaluate the stability of HCAs in urine and were analyzed with each batch of samples for 8 months. The recoveries of HCAs were above 80%, and RSDs were below 17.5% ($n = 23$), as shown in Fig. 7. Based on the improved method, urine samples from 1040 Korean school children were monitored. HCAs detected above the LOD in the samples were mainly Glu-P-1 and PhIP. The concentrations of Glu-P-1 and PhIP were found to be in the range of 0.05 to 1.26 ng/ml ($n = 139$) and 0.07 to 0.20 ng/ml ($n = 27$), respectively. Fig. 8 shows the SRM chromatograms of Glu-P-1 (m/z 199 $[M+H]^+ \rightarrow 181$) at 0.05 ng/ml and PhIP (m/z 225 $[M+H]^+ \rightarrow 210$) at 0.13 ng/ml in a positive urine sample. Quantification was achieved by using ratios of peak area of the individual HCAs to the corresponding 1-naphthylamine as the IS.

Conclusion

An effective sample handling and cleanup method to extract and purify HCAs from urine samples was optimized. Instead of multiple SPE elutions, LLE under basic conditions, followed by one-step SPE, was involved. The determination of trace-level HCAs was carried out by the triple quadrupole MS analyzer operating in SRM mode. These methodologies were able to detect lower concentrations of HCAs in urine samples when compared with the previous results of multiple SPE method with an ion trap mass analyzer. The whole procedure was validated by obtaining satisfactory accuracy and precision for most of analytes in this study. The results demonstrated the applicability of the improved method for the analysis of trace HCAs in urine for further epidemiological studies.

Acknowledgment

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