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Analytical method of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in human hair by column-switching liquid chromatography–mass spectrometry

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Abstract

A column-switching liquid chromatography–mass spectrometry was developed for quantification of 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) in human hair. Hair sample was digested in 1N NaOH at 100 °C, and PhIP was extracted using a Blue-Chitin column. The recovery rate was 73%, the limit of quantification was 50 pg/g hair, and intra-day and inter-day variations were 6.3 and 11.7%, respectively. PhIP was found in 42 of the 46 hair samples from 23 healthy volunteers: 110–3878 pg/g hair. The intrapersonal correlation between the first and second analyses was $r = 0.85$ (95% confidence interval, 0.65–0.94). A positive correlation was observed between PhIP levels and melanin content in hair. This study indicates the ability of this method to detect levels of PhIP in hair.

1. Introduction

Heterocyclic amines (HCAs) are formed during ordinary cooking, such as frying, grilling, broiling of meat and fish, and are thought to be involved in the causation of human cancer [1–4]. However, it has been difficult to estimate accurate exposure to HCAs by questionnaire survey in epidemiological studies. Estimates of the intake of meat and fish can not accurately predict HCA levels because the quantity of HCAs formed in food varies with cooking conditions. Therefore, suitable biomarkers of individual exposure are required to assess exposure. In previous studies, HCA levels in human urine have been investigated [5,6]. However, HCAs are rapidly eliminated from the body [7]. Hair is another possible specimen for biological monitoring. It has often been used for tests of drug abuse [8–10]. Drugs in hair have a long half-life compared with those in urine or blood, and hair samples are easily obtained. Thus, the HCA level in hair may be a possible biomarker of exposure. Our previous reports suggested that 2-amino-1-methyl-

6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3, 8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) were major compounds of HCAs to which the Japanese population is exposed [11,12]. The objective of the present study was to establish method for detecting HCAs in human hair samples by liquid chromatography–mass spectrometry (LC–MS) with a column-switching technique. This is the first report of the determination of PhIP and MeIQx in human hair using column-switching LC–MS.

2. Experimental

2.1. Reagent

PhIP, 2-amino-1-(trideuteromethyl)-6-phenylimidazo[4,5-*b*]pyridine (PhIP-d$_3$) and MeIQx, 2-amino-8-methyl-3-(trideuteromethyl)imidazo[4,5-*f*]quinoxaline (MeIQx-d$_3$) were obtained from Toronto Research Chemicals (Downsview, Ont., Canada). Sodium dodecyl sulphate (SDS) was purchased from Wako Pure Chemical (Osaka, Japan), Soluen350 from Packard (Meriden, CT, USA), and Sepia melanin from Sigma (St. Louis, MO, USA). Blue-Chitin

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column (Waters Sep-Pak cartridge filled with 120 mg of Blue-chitin containing 5 μmol dye) from Funakoshi (Tokyo, Japan) is a product specially developed for binding and recovering polycyclic carcinogens. Blue-chitin was prepared by binding copper phthalocyanine trisulfonate to chitin (poly-*N*-acetylglucosamine). Other reagents and solvents were HPLC analytical grade (Wako Pure Chemical, Osaka, Japan).

2.2. Standard solution and calibration curve

Stock solutions of PhIP, PhIP-d$_3$, MeIQx and MeIQx-d$_3$ were prepared in 100% methanol and stored at −20 °C. Standard solutions (40 μM ammonium acetate (pH 4):methanol, 1:1 (v/v)) for calibration were prepared in the concentration range 0.15–7 pg/μl, and stored at −20 °C until used. Calibration curves were made up as often as the analyses were done. The correlation coefficients of the calibration curves were better then 0.99. The levels of unknown samples were calculated from the peak height. To quantify PhIP and MeIQx in hair sample, the peak ratios of standards to internal standards were used for adjustment of the recovery.

2.3. Instrumental and chromatographic conditions

The column-switching liquid chromatography–mass spectrometry (LC–MS) system was LCMS-2010A coupled with a column switching system (Co-sense for BA system, Shimadzu, Kyoto, Japan). A SIL-10APvp automatic injector equipped with a contamination control kit (Shimadzu, Kyoto, Japan) was used. Three hundred microliter of the sample was injected by an auto-sampler, and was loaded onto the extraction column (Shim-pack MAYI-ODS column, 2.0 mm × 10 mm, Shimadzu, Kyoto, Japan) by 10 mM ammonium acetate at a rate of 2.0 ml/min for 4 min. Then, the valve was switched. The analyte was introduced into the analytical column (Mercury MS LUNA 3 μm C18 column, 2.0 mm × 20 mm, Penomenex, Torrance, CA, USA) at a rate of 0.2 ml/min. 40 μM ammonium acetate (pH 4.0, A) and methanol (B) were used as an analytical mobile phase. The gradient program was as follows: 13%B (0–5 min)–45%B (5.01 min)–45%B (5.01–13 min)–13%B (13.01 min). The column oven was maintained at 40 °C.

The mass spectrometry conditions for electrospray ionization (ESI)–MS were as follows: drying nitrogen gas temperature was set at 250 °C and introduced into the capillary at a flow rate of 4.5 l/min; the capillary was held at a potential of 4.5 kV for the positive ion mode. In selected ion monitoring (SIM) mode, ions at *m/z* 214, 217, 225 and 228 were assigned to [$M + H$]$^+$ of MeIQx, MeIQx-d$_3$, PhIP and PhIP-d$_3$, respectively.

2.4. Hair samples

Hair samples from 23 volunteers (8 males, 15 females) were used in this study. The volunteers gave their informed consent. Hair was obtained by normal hair cutting; approximately 3–5 g of hair was collected. Sampling was repeated after approximately 1–3 months. Samples were collected between March and September, then stored in paper envelopes at room temperature. Three grams of hair was washed in 0.1% SDS (100 ml) by ultrasonication for 5 min, after which the liquid was decanted, then washed four times with water (100 ml), once with 100% ethanol (50 ml), and dried at room temperature [13].

2.5. Extraction procedure

The dried hair sample was weighed, and 1N NaOH (100 ml) and 10 ng of internal standard (MeIQx-d$_3$, PhIP-d$_3$) were added. This solution was incubated at 100 °C for 45 min in a capped container (225 ml graduated conical tube with polypropylene cup, Falcon, NJ, USA), and the tube was capped loosely. After centrifugation at 3100 rpm for 10 min, the supernatant was filtrated (5B filter, ADVANTEC, Tokyo, Japan). The filtrate was neutralized to pH 7–9 with 6N HCl. HCAs in this filtrate were extracted using the Blue-Chitin column absorption method [14]. Briefly, HCAs absorbed on a Blue-Chitin column were eluted with 20 ml of MeOH–28% NH$_3$ (50:1) (flow rate, 5 ml/min). The eluate was concentrated under vacuum at room temperature using a centrifugal concentrator CC-105 with low temperature trap TV-105 (TOMY, Tokyo, Japan). The residue was dissolved in 1 ml of 100% MeOH. After centrifugation of 3100 rpm for 10 min, the supernatant was collected, and then was concentrated under vacuum. The residue was dissolved in 2 ml of 0.1N HCl. After washing with 2 ml of *n*-hexane, the aqueous layer was adjusted to pH > 10 with 28% NH$_3$, and then extracted twice with 2 ml of dichloromethane. The organic layer was concentrated under vacuum. The residue was dissolved in 500 μl of 40 μM ammonium acetate: 100% MeOH (1:1 (v/v)), and then filtrated with 0.45 μm filter (Ultrafree-MC, Millipore, Bedford, MA, USA).

2.6. Spectrophotometric characterization of melanin

It was shown that PhIP bound to melanin-rich tissues in rat [15], and other HCAs showed a strong affinity for melanin in autoradiographic studies in vivo [16,17]. Spectrophotometric characterization of melanin in hair samples was carried out according to a previous report [18–21]. Hair sample (1 mg) and sepia melanin (1 mg) were dissolved in 1 ml of a mixture of Soluene-350 and water (9:1 (v/v)), followed by heating at 95 °C for 45 min. Optical density was observed at 500 and 650 nm (A$_{500}$ and A$_{650}$) in Jasco V-550 (Japan Spectroscopic, Tokyo, Japan). A$_{500}$ indicates the quantity of total melanin, and the ratio A$_{650}$/A$_{500}$ equals the ratio of eumelanin to total melanin in hair sample. Total melanin concentrations of hair samples were calculated according to a previous method [12].
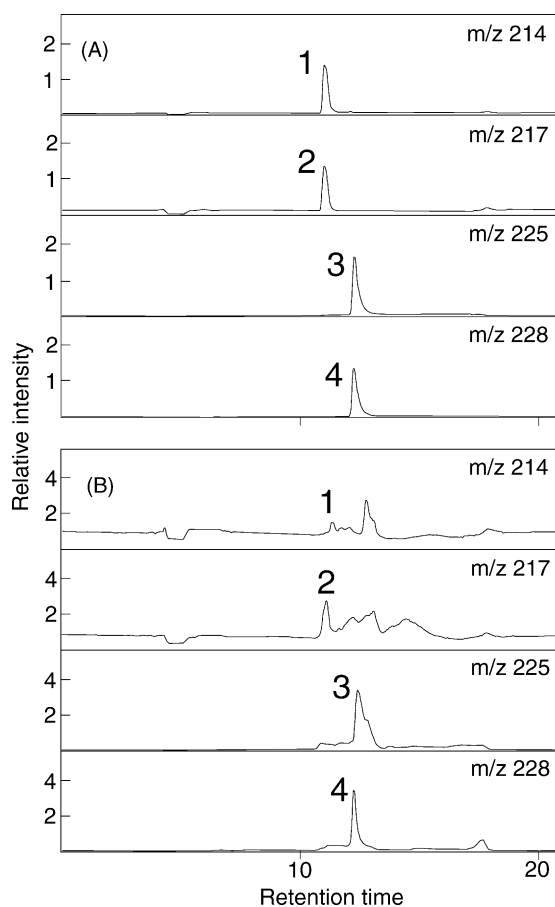
Fig. 1. LC–MS chromatogram. Panel A shows the standard compounds. Panel B shows an example of the analysis of hair (non-dyed hair, Subject D in Table 3). Peaks: 1, MeIQx; 2, MeIQx-d$_3$; 3, PhIP; 4, PhIP-d$_3$.

3. Results and discussion

3.1. Liquid chromatography–mass spectrometry

Using LC–MS with a pre-column system, we could detect PhIP and MeIQx in hair. A LC–MS chromatogram is shown in Fig. 1. The retention times of PhIP and MeIQx were 12.4 and 11.2 min, respectively. Panel A shows 7 pg/μl of each standard (PhIP, PhIP-d$_3$, MeIQx, MeIQx-d$_3$), and panel B presents the chromatograms of extracted hair sample (non-dyed hair, Subject D in Table 3).

3.2. Limit of detection, linearity and recovery

The background noise in the blank test was examined using solvent buffer (40 μM ammonium acetate: MeOH = 1:1). The instrumental limit of detection (LOD) and the limit of quantification (LOQ) were calculated as follows; average background + 3S.D., average background + 10S.D., respectively [22]. In the present system, LOD and LOQ were 6.9 and 13 pg, respectively. The detection limit for PhIP and MeIQx was 50 pg/g hair.

Table 1
Recovery of MeIQx, PhIP and their internal controls in this method

	Recovery (%, $n = 6$)	
	Mean	S.D.
MeIQx	37.3	1.5
MeIQx-d$_3$	36.0	2.7
PhIP	73.3	6.6
PhIP-d$_3$	74.8	6.9

Two ng of MeIQx, MeOQx-d$_3$, PhIP and PhIP-d$_3$ in 1N NaOH was examined under the extraction and analytical conditions described in the text.

Linearity of analysis was confirmed from the LOQ level to nanogram range. To determine the recovery rate, 2 ng of PhIP, PhIP-d$_3$, MeIQx and MeIQx-d$_3$ in 1N NaOH (100 ml) was extracted by the same method shown in Section 2. The quantification was done by the standard calibration curves. Recovery rates of the compounds in the Blue-Chitin column method are shown in Table 1. Recovery of PhIP and MeIQx were 73 and 37%, respectively. Recovery rates of each internal standard (PhIP-d$_3$, MeIQx-d$_3$) were similar (75 and 36%, respectively), indicating the appropriate use as internal standards.

3.3. Precision and accuracy

Table 2 shows the intra-day and inter-day coefficients of variation of the analyses. We used a quality control sample. PhIP showed acceptable precision (intra-day CV: 6.3%, inter-day CV: 11.7%) and accuracy (inter-day S.D.: 6.3%, inter-day S.D.: 11.7%). The intra-day variation of MeIQx was also acceptable (CV: 9.0%), but the inter-day variation was large (CV: 23.9%). Because the inter-day precision is considered to be a better indication of the precision, the MeIQx level was less reliable [23]. The poor MeIQx precision can be explained in part by instability during the extraction or wash process, although the loss of MeIQx could not be monitored during the extraction/washing process because we did not use an isotope-labeled internal control.

3.4. Interferences

Concerning MeIQx, an unknown peak (immediately after the MeIQx peak, m/z 214, retention time 11.5 min) interfered with quantification at the level <50 pg/g hair. This unknown

Table 2
Intra-day and inter-day variations of the analysis

	Intra-day ($n = 4$)			Inter-day ($n = 4$)		
	Mean (pg/g hair)	S.D.	CV (%)	Mean (pg/g hair)	S.D.	CV (%)
MeIQx	381.1	34.2	9.0	325.9	78.0	23.9
PhIP	1131.8	71.4	6.3	1234.2	145.0	11.7

The hair sample used in these analyses was from a male subject without hair coloring.

Table 3
Levels of MeIQx and PIhP in hair samples

	Subject	Hair-weight adjusted				Melanin-adjusted			
		MeIQx (pg/g hair)		PhIP (pg/g hair)		MeIQx (pg/g melanin)		PhIP (pg/g hair)	
		First	Second	First	Second	First	Second	First	Second
Male									
Hair-coloring (+)	A	ND	ND	2030	2651			45	66
Hair-coloring (−)	B	<LOQ	<LOQ	1712	1610			13	16
	C	<LOQ	<LOQ	3878	3469			34	32
	D	473	333	2534	1827	4	3	23	18
	E	549	301	3830	2586	5	3	32	25
	F	<LOQ	212	1480	2344		2	17	23
	G	<LOQ	234	1068	1004		2	11	10
	H	<LOQ	<LOQ	1574	1416			12	11
Female									
Hair-coloring (+)	I	ND	ND	705	843			14	11
	J	ND	ND	ND	ND				
	K	ND	ND	641	507			8	9
	L	ND	ND	246	110			3	2
	M	ND	ND	222	571			3	8
	N	ND	ND	ND	ND				
	O	ND	ND	680	1056			11	19
	P	ND	ND	1156	1355			16	17
	Q	ND	ND	1130	680			12	7
	R	ND	ND	449	315			7	5
	S	ND	ND	817	1446			10	18
Hair-coloring (−)	T	584	<LOQ	1211	2074	6		12	16
	U	275	223	1781	1225	5	4	33	25
	V	328	529	991	1683	2	4	7	12
	W	196	<LOQ	657	465	2		7	5

<LOQ indicates that MeIQx and/or PhIP concentration were lower than the limit of quantification. ND indicates not detectable.

peak was found only in dyed hair samples, so MeIQx in dyed hair samples could not be detected by this method. A shoulder peak with the PhIP did not interfere with its quantification.

3.5. Application

We measured PhIP and MeIQx levels in hair samples from 23 healthy volunteers. The results are shown in Table 3. PhIP was detected in 42 of 46 samples, and MeIQx in 12 of 46 samples. PhIP was not quantified in two subjects due to interference by unknown substances. The possible cause of interference by hair dye or other factors needs further exploration. Three grams of hair sample was used in the present study, but it was possible to measure the compound using hair sample >450 mg. The median level of PhIP detected in the hair samples was 1184 (110–3878) pg/g hair. This level is similar to the result in a previous study that used gas chromatography–mass spectrometry [24]. Distribution of the PhIP levels was log-normal. The intrapersonal correlation between the first and the second analyses was $r = 0.85$ (95% confidence interval [CI] = 0.65–0.94). The interpersonal variation was larger than the intrapersonal variation. Accurate hair cutting or shaving was needed for experimental studies to assess the time-dependent exposure. However, it was practically impossible in this study. The first hair sample showed former exposure compared with the second hair sample, if normal hair cutting was done. In another ward, two hair samples from one subject showed exposures at two different time points. This information is indispensable as a biomarker in future studies.

The melanin content in the hair samples ranged from 48.3 to 138.3 μg/g hair. The median level of PhIP adjusted by melanin levels was 12 (2–66) pg/g melanin. The intrapersonal correlation between the first and second analyses was $r = 0.86$ (95% CI = 0.67–0.94). A positive correlation was observed between PhIP levels in hair (pg/g hair) and melanin content in hair (μg/g hair) ($r = 0.40$, 95% CI = −0.03–0.83 in the first analysis; $r = 0.45$, 95% CI = 0.04–0.86 in the second analysis, Spearman rank correlation). These results were in accordance with the above experimental studies. These findings suggest that the melanin-adjusted level may be a better indicator than the crude level as the biomarker.

This method is only a first step in establishing a biomarker of HCA exposure. Although further improvement is needed with regard to the MeIQx level and dyed hair, the present study showed the ability of the analytical method and levels observed in healthy volunteers. Concerning the validity of the method as an epidemiological tool, we are going to

analyze the data from hair analysis, the dietary record and food frequency questionnaire.

4. Conclusion

The present study indicates the ability of this method to detect the PhIP levels in human hair.

Acknowledgements

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