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Analysis of heterocyclic amines as their N-dimethylaminomethylene derivatives by gas chromatography with nitrogen–phosphorus selective detection

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Abstract

A selective and sensitive method was developed for the determination of heterocyclic amines (HCAs) by gas chromatography (GC). HCAs were converted into their N-dimethylaminomethylene derivatives and measured by GC with nitrogen–phosphorus selective detection using two connected fused-silica capillary columns containing DB-1 and DB-17ht. The derivatives of the 10 HCAs were sufficiently volatile and stable to give single symmetrical peaks. The calibration curves for HCAs in the range 0.5–10 ng were linear with correlation coefficients being above 0.998. The detection limits at a signal-to-noise ratio of 3 were ca. 2–15 pg per injection.

Keywords: Derivatization, GC; N-Dimethylaminomethylene; Amines; Heterocyclic amines

1. Introduction

Heterocyclic amines (HCAs) formed during heating of amino acids, proteins, creatinine and sugars are potent mutagens in the Ames/*Salmonella* assay [1–8]. Up to the present, twenty-three HCAs have been isolated as mutagens, and the structures of nineteen of them were determined [8]. Many of these HCAs have been isolated from various proteinaceous foods including cooked meats and fish [4–7,9–12], and some of them have also been detected in environmental components such as airborne particles [13–15], indoor air [14,16], cigarette smoke [14,16–18], diesel exhaust particles [15,19], cooking fumes [20,21] and rain water [13,22]. Moreover, some HCAs have been detected in biological samples such as urine, plasma, bile and feces [9,23–26]. These results suggest that humans are continually exposed to HCAs in a number of ambient environments. On the other hand, some of these mutagenic HCAs have been verified to be carcinogenic to rodents [1,4–7,12,27,28] and non-human primates [28,29], and to be implicated in human carcinogenesis [30,31]. Moreover, recent investigations revealed that HCAs also possess cardiotoxic effect [28] and various pharmaco-toxicological activities such as convulsant activities [32,33] and potent inhibitory effects on platelet function and dopamine metabolism [33–36]. Therefore, the isolation and measurement of HCAs in the environment are very important for human health risk assessment. It is a matter of urgency to develop a useful method for monitoring the exposure levels of HCAs.

The determination of HCAs has been carried out

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by enzyme-linked immunosorbent assay [37], capillary zone electrophoresis [22,38], high-performance liquid chromatography with UV [9,21,25,39–44], electrochemical [9,41,45–47] and fluorescence [9,13–16,18,19,21,24,25,40,41,48] detection, liquid chromatography–mass spectrometry [49–52] and gas chromatography–mass spectrometry with selected-ion monitoring [20,23,26,53–55]. Knize et al. [56] has recently reviewed these methods used for the analysis of HCAs in cooked foods. Although some of these methods are highly sensitive and selective, many of them require sophisticated and expensive equipment that is beyond the reach of many laboratories. Furthermore, most of these methods are restricted to the determination of a selected group of HCAs, and require time-consuming separation.

On the other hand, gas chromatography (GC) has been widely utilized for amine analysis because of its inherent advantages of simplicity, high resolving power, high sensitivity and low cost [57]. However, the main problem is the need to derivatize into less polar compounds because of its strong adsorption. Acetic, trifluoroacetic and heptafluorobutyric anhydrides, pentafluorobenzyl bromide (PFB-Br), 3,5-bis-trifluoromethylbenzyl bromide (bis-TFMBZ-Br) and bis-trifluoromethylbenzoyl bromide (bis-TFMBO-Cl) have been tested as derivatization agents for some HCAs [20,23,26,45,53–55]. However, acylation with acid anhydrides yielded derivatives with very poor GC properties. Although the alkylation products with PFB-Br, bis-TFMBZ-Br and bis-TFMBO-Cl had good GC properties for some HCAs, these methods gave a mixture of mono- and di-alkylated forms. Incomplete derivatization leads to non-reproducible results. Consequently, a useful GC method has not yet been developed.

N,N-Dimethylformamide dimethyl acetal (DMF-DMA) has been used not only for methyl esterification of carboxylic acid [58] but also for one step derivatization of amino acids into N,N-dimethylaminomethylene methyl esters [59]. The reaction with amino group is based on the Schiff base condensation of primary amines. Therefore, it is considered that each HCA gives a single derivative by the reaction with DMF-DMA. In the present work, we investigated a convenient and reliable method for the simultaneous determination of HCAs by GC with nitrogen–phosphorus selective detection (NPD) after simple derivatization with DMF-DMA.

2. Experimental

2.1. Reagents

The heterocyclic amines (HCAs) used in this study are listed in Fig. 1. 2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) was purchased from Toronto Research Chemicals (Downsview, Canada). 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinox-

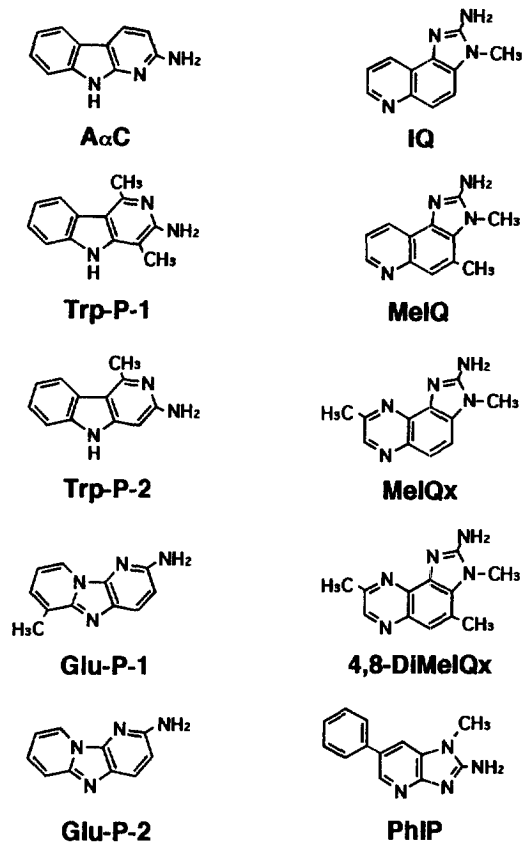

Fig. 1. Structures of heterocyclic amines used in this study. AαC=2-amino-9H-pyrido[2,3-*b*]indole, Trp-P-1=3-amino-1,4-dimethyl-5H-pyrido[3,4-*b*]indole, Trp-P-2=3-amino-1-methyl-5H-pyrido[3,4-*b*]-indole, Glu-P-1=2-amino-6-methyldipyrido[1,2-*a*:3′2′-*d*]imidazole, Glu-P-2=2-aminodipyrido[1,2-*a*:3′2′-*d*]imidazole, IQ=2-amino-3-methyl-imidazo[4,5-*f*]quinoline, MeIQ=2-amino-3,4-dimethylimidazo[4,5-*f*]-quinoline, MeIQx=2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, 4,8-DiMeIQx=2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline, PhIP=2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

aline (4,8-DiMeIQx) and 2-amino-9H-pyrido[2,3-*b*]indole (AαC) were purchased from Funakoshi Pharmaceutical (Tokyo, Japan). 3-Amino-1,4-dimethyl-5H-pyrido[3,4-*b*]indole (Trp-P-1) was purchased from Wako Pure Chemical Industries (Osaka, Japan). 3-Amino-1-methyl-5H-pyrido[3,4-*b*]indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-*a*:3′2′-*d*]-imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3′2′-*d*]-imidazole (Glu-P-2) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) were kindly provided by Dr. H. Hayatsu, Professor of Okayama University. 2-Amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline (4,7,8-TriMeIQx; Funakoshi) was used as an internal standard (I.S.). Each HCA was dissolved in methanol to make a stock solution at a concentration of 0.1 mg/ml and used after dilution with methanol to the require concentration. N,N-Dimethylformamide dimethyl acetal (DMF-DMA) was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical-reagent grade.

2.2. Derivatization procedure

An aliquot of the sample containing 0.5–10 ng of HCAs was pipetted into a 10-ml Pyrex glass tube with a PTFE-lined screw-cap. 10 ng of I.S. and 10 μl of DMF-DMA was added to this solution, and the mixture (0.1–0.5 ml) was heated at 100°C for 15 min without capping the tube. After evaporation to dryness, the residue was dissolved in 20–40 μl of ethyl acetate and then 1 μl of this solution was injected into the gas chromatograph.

2.3. Gas chromatography

GC analysis was carried out with a Hewlett-Packard 5890 Series II gas chromatograph equipped with an electronic pressure control (EPC) system, a split/splitless capillary inlet system and a nitrogen–phosphorus detector (NPD). Two connected fused-silica capillary columns (J&W, Folsom, CA, USA) containing, respectively, DB-1 (10 m×0.25 mm I.D., film thickness 0.25 μm) and DB-17ht (10 m×0.25 mm I.D., film thickness 0.15 μm) with a two-way press fit fused-silica tube were used. The operating conditions were as follows: column temperature, programmed at 10°C/min from 230 to 280°C, programmed at 25°C/min from 280 to 330°C and held at 330°C for 1 min; injection and detector temperature, 340°C. The inlet helium pressure (flow-rate) controlled with EPC, was programmed at 10 kPa/min from 180 (2.05 ml/min) to 230 kPa (2.5 ml/min), programmed at 25 kPa/min from 230 (2.5 ml/min) to 280 kPa (2.9 ml/min) and held at 280 kPa (2.9 ml/min) for 1 min. Make-up gas flow-rate: 30 ml/min; split ratio: 10:1. A chromatographic run (run made with no sample injected) data was subtracted from sample run data to remove baseline drift (usually caused by column bleed) using a single-column compensation function and then base-line corrected data was recorded on the chromatogram. The peak height ratios of HCAs and the I.S. were measured and the peak height ratios against the I.S. were calculated to construct calibration curves.

2.4. Gas chromatography–mass spectrometry (GC–MS)

A Hewlett-Packard Model 5890A gas chromatograph was operated in conjunction with a VG Analytical Model 70-SE mass spectrometer and a VG-11-250J mass data system. A fused-silica capillary column containing cross-linked OV-210 (Quadrex, New Haven, CT, USA, 6 m×0.25 mm I.D., film thickness 0.25 μm) was used. Column temperature: programmed at 5°C/min from 140 to 270°C; injection temperature, 270°C; ion-source temperature, 270°C; ionizing voltage, 40 eV; helium flow-rate, 1 ml/min.

3. Results and discussion

Most of HCAs are polar and less volatile, and tend to elute as broad and tailing peaks due to the strong adsorption during GC analysis. Therefore, they can not be detected in low concentration. Derivatization of amines may be employed not only to reduce the polarity but also to improve the volatility, selectivity, sensitivity and separation of these amines [57]. The commonly used derivatization reactions such as acylation, alkylation and silylation for GC analysis of amines were tested for the derivatization of HCAs. Acylation with trifluoroacetic and propionic anhydrides and trimethylsilylation with N,O-bis-(trimethylsilyl)trifluoroacetamide gave poor GC properties or no peaks. Ethoxycarbonylation with ethyl chloroformate gave good GC properties for

some HCAs, but PhIP was not detected. Among several derivatization reagents tested, DMF-DMA proved to be the most satisfactory GC properties for all HCAs. DMF-DMA forms a Schiff base-type derivative with primary amine [60,61]. Experiments were conducted to find suitable reaction conditions for the preparation of N-dimethylaminomethylene derivatives of HCAs. The reaction with DMF-DMA proceeded in anhydrous methanol solution. As shown in Fig. 2A–C this reaction was completed within 10 min at 100°C by using 10 μl of DMF-DMA. Amino pyrido compounds such as AαC, Trp-P-1 and Glu-P-1 tended to depend on the reaction temperature as compared to amino imidazo compounds such as IQ, MeIQx and PhIP. The other four HCAs could be also derivatized under the same optimum reaction conditions. Most of excess reagent was removed by evaporation.

The structures of the HCA derivatives were confirmed by GC–MS analysis. As shown in Fig. 3, a molecular ion peak $[M]^+$ was observed for each of the derivatives and other common ion peaks which were useful for structure elucidation, were $[M-15]^+$ (CH_3), $[M-44]^+$ $[N(CH_3)_2]$, $[M-56]^+$ $[C=N(CH_3)_2]$ and $[M-71]^+$ $[N=CHN(CH_3)_2]$. Although a small $[M]^+$ was observed for Glu-P-1 and Glu-P-2, the abundant fragment (base peak) was $[M-1]^+$ and all further fragments were originated from $[M-1]^+$, such as m/z=237, 208, 196 and 181 for Glu-P-1. These results suggest that the imidazole ring of Glu-P-1 and Glu-P-2 is responsible for the cleavage resulting in the formation of dipyridylamine compounds ($[M-1]^+$). The derivatives of HCAs were stable under normal laboratory conditions and no decomposition was observed during GC analysis.

In preliminary tests for several capillary columns at constant flow mode, DB-1 and DB-17ht gave the best separation for HCAs, although AαC and Glu-P-2 were overlapped and some pairs of HCAs were partially overlapped, e.g., MeIQ/MeIQx in the DB-1 column and Glu-P-2 and AαC/Glu-P-1 in the DB-17ht column. In order to solve this problem, we tried to connect both columns and EPC programming. Of several GC conditions tested, the connection of two columns (10 m DB-1 and 10 m DB-17ht), the two-ramp temperature programme and EPC programme given in Section 2.3 resulted in improved separation of Glu-P-1, MeIQ and MeIQx, but the overlapping of AαC and Glu-P-2 could not be improved. As shown in Fig. 4, ten HCAs could be well separated as single and symmetrical peaks within 7 min by using a capillary column connected DB-1 and DB-17ht, except for the overlapping of peaks of AαC and Glu-P-2.

The derivatives of HCAs gave an excellent response in the NPD due to the excess of nitrogen. As

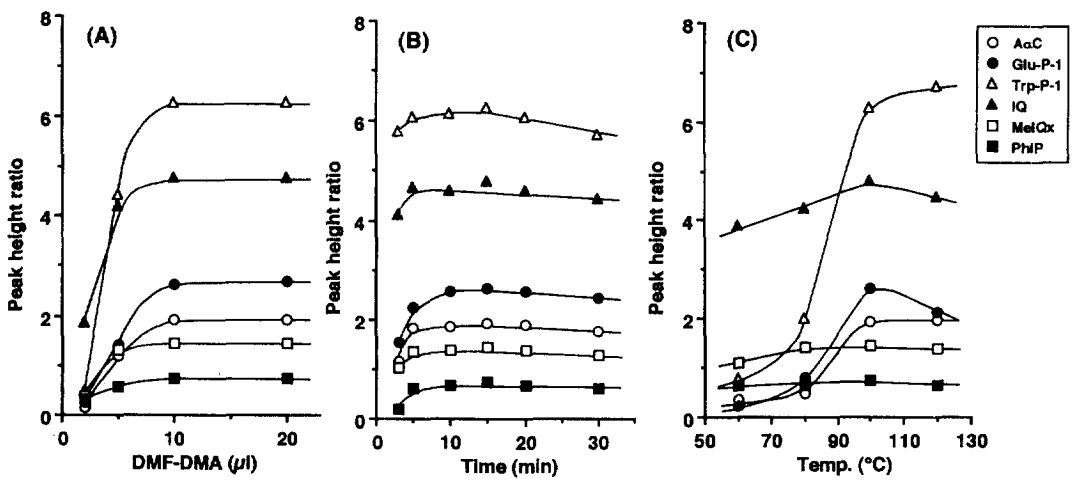

Fig. 2. Effects of (A) amount of N,N-dimethylformamide dimethyl acetal, (B) reaction time and (C) temperature on the conversion of heterocyclic amines into their N-dimethylaminomethylene derivatives.

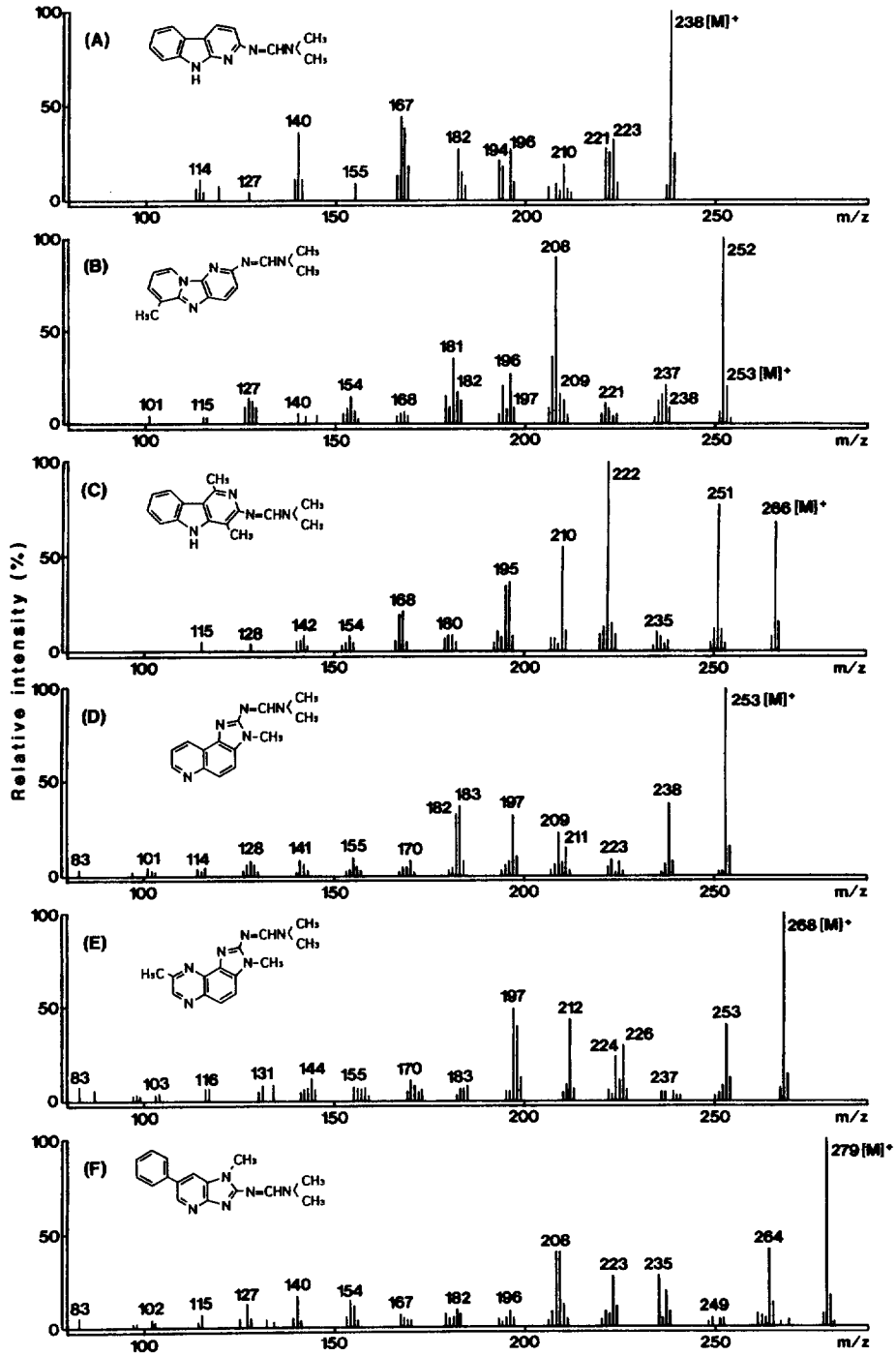

Fig. 3. Mass spectra of the N-dimethylaminomethylene derivatives of heterocyclic amines. (A) AαC, (B) Glu-P-1, (C) Trp-P-1, (D) IQ, (E) MeIQx, (F) PhIP.

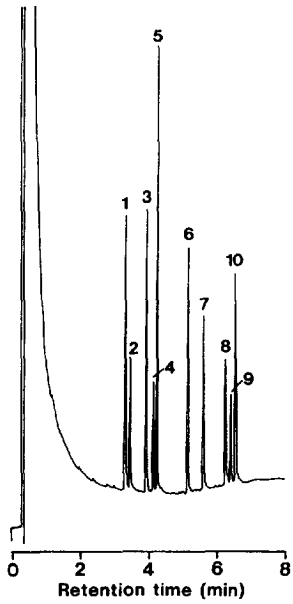

Fig. 4. Typical gas chromatogram obtained from standard heterocyclic amines (containing 5 ng of each amine). GC conditions are given in Section 2.3. Peaks: 1=AαC+Glu-P-2, 2=Glu-P-1, 3=Trp-P-1, 4=Trp-P-2, 5=IQ, 6=MeIQ, 7=MeIQx, 8=4,8- DiMeIQx, 9=PhIP, 10=4,7,8-TriMeIQx (I.S.).

shown in Table 1, the minimum detectable amounts of these nitrogen-rich HCAs at a signal-to-noise ratio of 3 under our instrumental conditions were 2–15 pg as injection amount. The calibration curves for HCAs were conducted using 4,7,8-TriMeIQx, which showed similar behaviour to other HCAs during the derivatization and was well separated from other HCAs on a chromatogram as the I.S. Various amounts of HCAs ranging from 0.5 to 10 ng were derivatized, and aliquots representing 25–250 pg were injected into the GC–NPD. As shown in Table 1, a linear relationship was obtained from both logarithmic plots of the peak height ratios and the HCA amounts with correlation coefficients of above 0.998. The relative standard deviations of peak height ratios for each HCA in each point were 0–9.8% (n=3) except for 0.5 ng level being 2.8–15.4%.

4. Conclusion

These experiments have conclusively demonstrated that HCAs can be accurately and precisely determined by GC–NPD as their N-dimethylaminomethylene derivatives. This method is simple, rapid, selective and sensitive, and ten HCAs can be simultaneously and quantitatively analysed within 30 min. We believe that this method provides a useful tool for environmental analysis. Further investigations on the application of this method to environmental samples are in progress.

Table 1
Linear regression data and detection limits for heterocyclic amines

Heterocyclic amine	Regression line[a]		Correlation coefficient (r)	Number of data (n)	Detection limit (pg)
	Slope (a)	Intercept (b)			
AaC	1.291	−0.998	0.9989	15	9
Glu-P-2	1.197	−1.155	0.9986	15	14
Glu-P-1	1.194	−1.019	0.9994	15	8
Trp-P-1	1.143	−0.667	0.9988	15	3
Trp-P-2	1.335	−1.239	0.9994	15	14
IQ	1.093	−0.457	0.9982	15	2
MeIQ	1.084	−0.696	0.9980	15	4
MeIQx	1.208	−0.952	0.9989	15	8
4,8-DiMeIQx	1.217	−1.095	0.9989	15	10
PhIP	1.259	−1.302	0.9995	15	15

[a] Range: 0.5–10 ng; log $y=a\log x+b$.

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