Journal of Chromatography, 529 (1990) 125–133
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5275

Simultaneous determination of amino-α-carbolines and amino-γ-carbolines in cigarette smoke condensate by high-performance liquid chromatography

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(First received September 12th, 1989; revised manuscript received February 15th, 1990)

ABSTRACT

A method for the simultaneous detection of amino-α-carbolines (2-amino-α-carboline and 2-amino-3-methyl-α-carboline) and amino-γ-carbolines (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole) by high-performance liquid chromatography has been developed. It consists of a three-step purification using three different columns with fluorometric detection. With this method, we have demonstrated that both amino-α-carbolines and amino-γ-carbolines are present in cigarette smoke condensate. The method may be useful for detecting these carcinogens in various materials.

INTRODUCTION

AαC (2-amino-α-carboline) and MeAαC (2-amino-3-methyl-α-carboline) were first isolated from pyrolysate of soybean globulin [1]. Later, these amino-α-carbolines were shown to be present not only in cooked foods such as grilled meat and chinese mushroom, but also in cigarette smoke condensate [2,3]. On the other hand, the amino-γ-carbolines, Trp-P-1 (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole) and Trp-P-2 (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole), were isolated from tryptophan pyrolysates as compounds with potent mutagenic effects to *Salmonella typhimurium* tester strains [4]. Later,

0378-4347/90/$03.50

not only the amino-γ-carbolines but also the amino-α-carbolines were shown to be carcinogenic to experimental animals [5]. Recent investigations revealed that the amino-γ-carbolines are present not only in the environment, including cooked foods and the air, but also in biological samples such as human plasma and dialysis fluid of patients with uremia [6–10]. We have previously developed a sensitive high-performance liquid chromatographic (HPLC) method for detecting amino-γ-carbolines, Trp-P-1 and Trp-P-2 [8–10]. In the course of the determination of the amino-γ-carbolines in various samples, we found that amino-α-carbolines were also detectable by a slightly modified version of our HPLC method. This paper describes the method for simultaneously isolating and quantitating amino-α-carbolines and amino-γ-carbolines.

EXPERIMENTAL

Materials

AαC (2-amino-α-carboline or 2-amino-9*H*-pyrido[2,3-*b*]indole) and MeAαC (2-amino-3-methyl-α-carboline or 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole) were kindly provided by Dr. K. Wakabayashi, National Cancer Center Research Institute (Tokyo, Japan). Trp-P-1 (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole) and Trp-P-2 (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole) were obtained from Wako (Osaka, Japan). The structures of these compounds are shown in Fig. 1. HPLC-grade acetonitrile, methanol and chloroform were purchased from Wako. All other chemicals were of analytical grade.

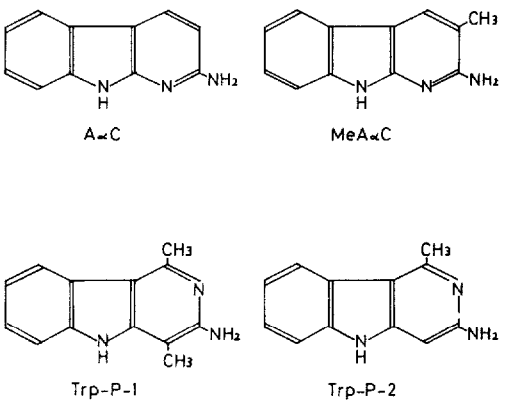

Fig. 1. Structures of amino-α-carbolines and amino-γ-carbolines. Abbreviations: AαC, 2-amino-9*H*-pyrido[2,3-*b*]indole; MeAαC, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole; Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole.

Preparation of cigarette smoke condensate

Commercial cigarettes (two Japanese brands, one American and one English) were used in this study. The filter-tipped cigarettes were smoked by an automatic smoking machine under standard conditions (puff frequency, 1 puff per 60 s; puff volume, 35 ml per 2 s; butt length, 30 mm) [3]. The cigarette smoke condensate was collected on glass-fibre filters (Toyo Roshi Type GA 200, Toyo Roshi, Tokyo, Japan) and extracted once with 100 ml of methanol–28% (50:1, v/v) ammonia water in an ultrasonic bath. The extract was evaporated to dryness under a nitrogen stream. The cigarette smoke condensate thus obtained was dissolved in 20 m*M* H_3PO_4 (pH 2.0)–acetonitrile (90:10, v/v) and filtered with a disposable filter unit (0.45 μm, Gelman Science Japan, Tokyo, Japan). The filtrate was used for HPLC analyses.

Apparatus

All chromatographic experiments were carried out by means of a Hitachi 655A chromatograph (Hitachi, Tokyo, Japan) equipped with an F-1000 fluorometric detector (Hitachi).

Liquid chromatographic conditions

In order to isolate amino-α-carbolines and amino-γ-carbolines completely, a three-step purification with HPLC was needed. Partial purification was carried out with an Asahipack ES-502C column (100 mm×7.6 mm I.D., 9.0 μm particle size; Asahi, Kawasaki, Japan) under the following conditions: mobile phase, 20 m*M* H_3PO_4 (pH 2.0)–acetonitrile (90:10, v/v); flow-rate 1.0 ml/min at 40°C [10]. The fluorescence was monitored at 399 nm, and the excitation wavelength was 266 nm. After injection of extracts from cigarette smoke condensate, the fraction corresponding to AαC/Trp-P-2 and the fraction corresponding to MeAαC/Trp-P-1 were collected separately. The volume of these fractions was reduced to ca. 1 ml with a centrifugal evaporator EC-57 (Sakuma Seisakusyo, Tokyo, Japan).

In the second step of the purification, the condensed fractions (ca. 1 ml) were further purified with a Nucleosil $5C_8$ column (150 mm×4 mm I.D., 10 μm particle size; Union, Takasaki, Japan) [10]. The solvent system for the fraction corresponding to AαC/Trp-P-2 of the first-step purification was 20 m*M* H_3PO_4 (pH 2.0)–acetonitrile (90:10, v/v), and the eluate for the MeAαC/Trp-P-1 fraction was 20 m*M* H_3PO_4 (pH 2.0)–acetonitrile (85:15, v/v). A flow-rate of 1.0 ml/min at 50°C was used. The fluorescence was monitored at 399 nm, and the excitation wavelength was 266 nm.

The final HPLC analysis was carried out on a Kaseisorb LC ODS-300-5 column (250 mm×7.5 mm I.D., 5 μm particle size and 300 Å pore size; Tokyo Chemical Industries, Tokyo, Japan). The mobile phase was a linear gradient (0–30%, v/v) of acetonitrile in 10 m*M* H_3PO_4 over 35 min [10]. The flow-rate was 3.0 ml/min at 50°C. The fluorescence was monitored as described above.

Calculations

Calibration curves were obtained by plotting peak heights (mm) as the y-axis and the amounts (ng) in the extract of cigarette smoke condensate as the x-axis. Regression analysis gave the best values for b and a in the equation $y=a+bx$. Cigarette smoke condensate spiked with amino-α-carbolines and amino-γ-carbolines was analysed by the same procedures. From the measured peak heights from the cigarette samples, the amounts of these compounds were calculated from the above linear equation.

Spectrophotometric analyses

Spectrometric measurements were performed on a Shimadzu UV 260 spectrophotometer (Shimadzu, Kyoto, Japan) and a Hitachi F-3000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) after the samples had been dissolved in methanol. The mass spectral analyses were conducted in the electron-impact (EI) mode using the direct insertion probe on a double-focusing JEOL JMS-Dx 300 mass spectrometer. The mass spectra were recorded with the JMA-3500 mass data analysis system, employing a 70-eV ionization voltage, 300-μA ionization current and 3-kV accelerating voltage with a source.

RESULTS AND DISCUSSION

Typical results obtained with cigarette smoke condensate are shown in Figs. 2 and 3. A chromatogram of the first-step purification is shown in Fig. 2A, where a quarter of the extract from the mainstream smoke of a Japanese filter cigarette was analysed. After the second purification step, the fractions corresponding to AαC, Trp-P-2 and MeAαC/Trp-P-1 were collected separately (Fig. 2B and C); and condensed to ca. 2 ml. These condensed fractions were analysed separately. Note that only one tenth of the AαC fraction was analysed owing to the high concentration of AαC. As shown in Fig. 3, sharp peaks corresponding to authentic compounds were clearly identifiable. The detection limit for Trp-P-1 was greater than 15 fmol (3.17 pg), that for Trp-P-2 was greater than 20 fmol (3.94 pg) [9,10], and those for AαC and MeAαC were greater than 60 fmol (11.0 pg for AαC and 11.82 pg for MeAαC). The detection sensitivities for Trp-P-1 and Trp-P-2 were apparently higher than those for amino-α-carbolines because the fluorometric detection conditions were set to be suitable for detecting amino-γ-carbolines.

To confirm the identity of the purified compounds, the fractions corresponding to authentic compounds were collected separately and pooled. The contents of each pooled fraction corresponding to AαC, MeAαC, Trp-P-1 and Trp-P-2 were extracted with chloroform [9,10], and the extracts were analysed spectrometrically. The compounds purified with HPLC were further confirmed to be amino-α-carbolines and amino-γ-carbolines by their absorbance and fluorescence spectra and mass spectra (not shown).

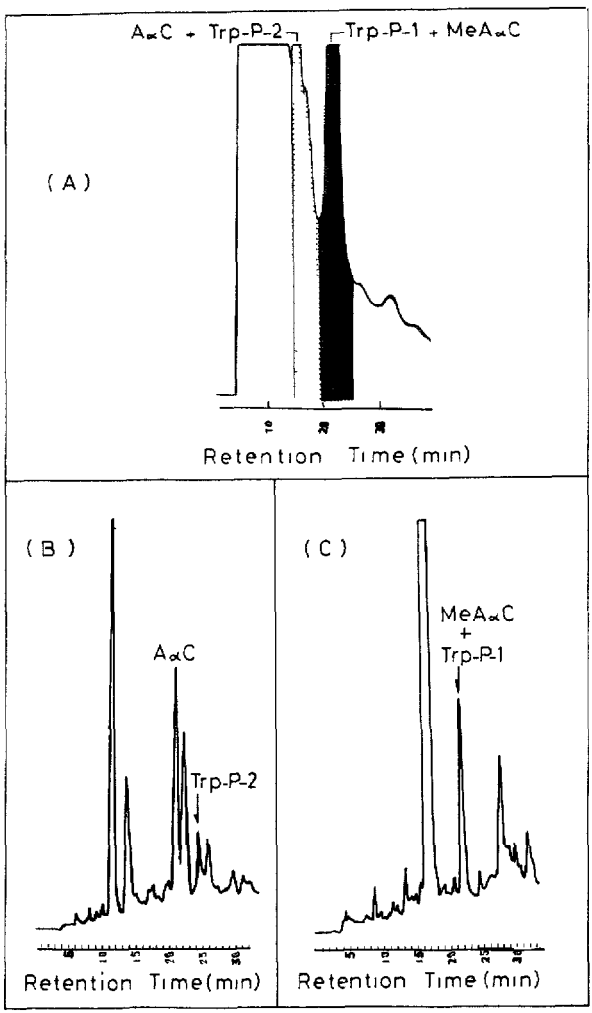

Fig. 2. Chromatogram of preparative and second-step purification of amino-α-carbolines and amino-γ-carbolines in cigarette smoke condensate. (A) Preparative chromatogram of cigarette smoke condensate on an ES-502C column. The fractions that appeared to correspond to AαC/Trp-P-2 and MeAαC/Trp-P-1 were collected separately. (B, C) Chromatograms of the second-step purification on a Nucleosil $5C_8$ column. The fractions corresponding to AαC/Trp-P-2 and MeAαC/Trp-P-1 in the first purification step were analysed separately.

Data from the calibration curves of peak height versus amounts of authentic reference compounds are shown in Table I. Table I also shows the analysis of variance between the extracted and non-extracted standard curves. The results indicate that the extracted and non-extracted standard curves are par-

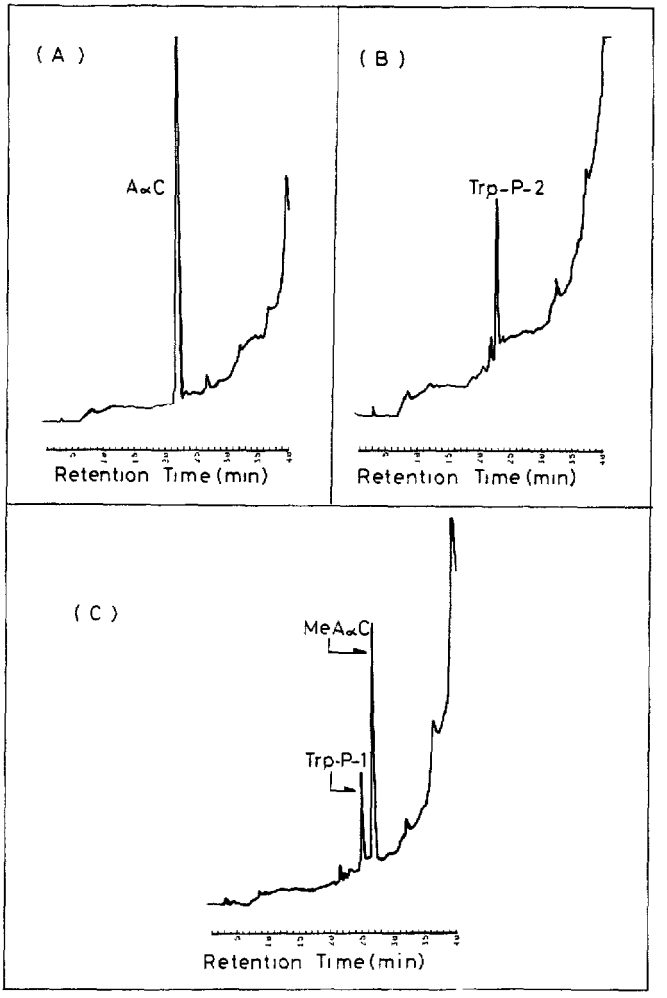

Fig. 3. Final chromatographic profiles of cigarette smoke condensate on an ODS-300-5 column. The fractions corresponding to AαC, Trp-P-2 and MeAαC/Trp-P-1 in the second purification step were analysed separately. (A) Final chromatogram of the AαC fraction. (B) Final chromatogram of the Trp-P-2 fraction. (C) Final chromatogram of the MeAαC/Trp-P-1 fraction.

allel. Therefore, the amounts of these compounds in cigarette smoke condensate were calculated using the above linear equation.

Recovery experiments were conducted as follows. The amounts of amino-α-carboline and amino-γ-carbolines in a cigarette smoke sample were determined. A sufficient amount of these compounds in a small volume was then added to the condensate to double the amount present. The spiked sample was redetermined. The difference between the first and second determinations was divided by the added amount to give the data shown in Table II.

TABLE I

LINEAR REGRESSION ANALYSES OF CALIBRATION CURVES

Compound	r	Slope	Intercept	Analysis of variance[a] [$F(6,1,0.005)=18.6351$]
Trp-P-1				
Non-extracted standard[b]	0.999	1.246	−0.256	$F_o=0.098$
Extracted standard[c]	0.991	1.220	44.308	
Trp-P-2				
Non-extracted standard	0.999	0.605	−0.707	$F_o=0.515$
Extracted standard	1.0	0.609	20.000	
AαC				
Non-extracted standard	0.999	0.256	−1.213	$F_o=0.310$
Extracted standard	0.999	0.255	12.817	
MeAαC				
Non-extracted standard	0.999	0.211	−0.171	$F_o=1.046$
Extracted standard	0.999	0.206	19.049	

[a] F_o = observed value following F distribution variance ratio ($V_{\text{sample preparation}}/V_{\text{error}}$); $F(f_1, f_2,\alpha)$ = density function of F distribution with f_1 and f_2 degrees of freedom.
[b] Standards dissolved in mobile phase.
[c] The extract from cigarette smoke condensate with known amounts of authentic standards.

TABLE II

RECOVERIES OF AUTHENTIC STANDARDS FROM CIGARETTE SMOKE CONDENSATE

Amount added (ng)	Mean recovery ($n=4$) (%)			
	Trp-P-1	Trp-P-2	AαC	MeAαC
0.2	66.4	-	-	-
0.4	65.1	-	-	-
1	-	61.9	-	-
2	-	63.2	-	69.2
4	-	-	-	66.1
25	-	-	62.6	-
50	-	-	61.8	-

The amounts of amino-α-carbolines and amino-γ-carbolines in cigarette smoke condensate are shown in Table III. The levels of amino-α-carbolines in mainstream smoke were remarkably higher than those of amino-γ-carbolines (Table III). In a previous report, the mean levels of AαC and MeAαC in cigarette mainstream smoke were reported to be 101.1 and 18.2 ng per cigarette, respectively [3]. Though we cannot precisely compare the amino-α-carboline levels in cigarette smoke with those in the previous report because of different

TABLE III

AMINO-α-CARBOLINES AND AMINO-γ-CARBOLINES IN CONDENSATE OF CIGARETTE MAINSTREAM SMOKE

Tobacco product	n^a	Carcinogenic heterocyclic amines (ng per cigarette)			
		Trp-P-1	Trp-P-2	AαC	MeAαC
Japanese filter cigarette A	3	0.29	1.03	27.70	2.21
Japanese filter cigarette B	3	0.46	0.97	44.81	2.44
Japanese filter cigarette C	4	0.48	1.10	47.21	2.94
U.S.A. filter cigarette	3	0.30	0.82	42.62	2.01
U.K. filter cigarette	3	0.36	0.84	47.83	2.90
Mean $\pm$ S.D.		0.38 $\pm$ 0.09	0.95 $\pm$ 0.12	42.03 $\pm$ 8.27	2.50 $\pm$ 0.41

[a]Number of cigarettes tested.

sample sources, the values of AαC and MeAαC obtained in this study (Table III) were apparently lower than those reported previously [3].

Amino-α-carbolines and amino-γ-carbolines are structurally very similar (Fig. 1). The amounts of these compounds in foods and biological samples are at the trace level [6–10]. Furthermore, there are many compounds that structurally resemble these compounds in the environment. For example, β-carbolines, such as harmane and tetrahydro-β-carboline, are widely distributed [11]. This may explain why it has been very hard to detect these carcinogens directly. In the present study, we have demonstrated that minute amounts of amino-α-carbolines (AαC and MeAαC) and amino-γ-carbolines (Trp-P-1 and Trp-P-2) can be determined simultaneously by HPLC. Our method may be useful in detecting these carcinogenic compounds in various samples such as foodstuffs.

ACKNOWLEDGEMENTS

The authors thank Dr. K. Wakabayashi, National Cancer Center Research Institute (Tokyo, Japan) for kindly providing the amino-α-carbolines. This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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