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Tetrahydro-β-carboline-3-carboxylic acids and contaminants of L-tryptophan

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Abstract

Methods for the separation, identification, and quantitative assay of contaminants of L-tryptophan implicated in eosinophilia-myalgia syndrome (EMS) are described. Propylsulfonic acid (PRS), benzenesulfonic acid (SCX), and octyl-derivatized silica (C_8) bonded-phase cartridges were used for the separation; LC–MS and GC–MS for identification; and HPLC–UV–fluorescence detection for quantitative analyses of norharman, harman, tetrahydro-β-carboline-3-carboxylic acid (TCCA), 1-methyltetrahydro-β-carboline-3-carboxylic acid (MTCA), 1,1′-ethylidenbis(tryptophan) (EBT), and 3-(phenylamino)alanine (PAA). The tissue distribution, excretion, and metabolism of these contaminants of L-tryptophan associated with EMS after acute and chronic dosage regimens are described. Considerable amounts of EBT were observed in the large intestine of rats administered EBT, showing a transfer without decomposition in gastric fluid. In addition, MTCA was detected in the blood and urine as well as the organs of rats treated with EBT, suggesting MTCA as a major metabolite of EBT. PAA accumulated markedly in the brain, among the organs of rats, after both acute and chronic administration of PAA, while MTCA accumulated in the kidneys of rats after chronic dosage of MTCA. Ethanol and/or acetaldehyde-induced formation of MTCA, as well as tryptophan-induced formation of TCCA, occurred endogenously in man and animals.

Keywords: Tetrahydrocarbolinecarboxylic acids; Tryptophan; Carboxylic acids; Ethylidenebis(tryptophan); (Phenylamino)alanine; Amino acids

1. Introduction

Eosinophilia-myalgia syndrome (EMS) is a newly recognized illness that has been connected to the intake of the amino acid L-tryptophan. Among the subjects for whom the source of the tryptophan was known, 29 of 30 case patients and 21 of 35 controls had consumed tryptophan manufactured by a single company [1]. Edema of the extremities, frequently accompanied by pruritus, paresthesia, and myalgia, scleroderma, fasciitis developed in the patients. All patients had blood eosinophilia in the acute phase of illness (1×10^9 cells/l) [2]. By the end of 1989 more than 1511 cases of EMS in 52 states of the USA, of which 38 were fatal. These incidences were followed by systematic investigations by the Centers for Disease Control (CDC), Food and Drug Administration (FDA), and other laboratories to elucidate the nature and mechanism of this hitherto recently described pathology.

The implicated L-tryptophan was shown to contain more than 60 impurities [3], some of which were proposed to contribute to EMS [4–8]. A compound designated as ''peak E'' was the first suggested to be responsible for EMS [1]. Subsequently, the structure of peak E was proposed as ditryptophan aminal

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of acetaldehyde (DTAA), *N,N′*-ethylidenebis-(tryptophan) [4,9] and then identified to be 1,1′-ethylidenebis(tryptophan) (EBT) [10,11]. EBT is instable in acidic solutions and readily decomposes to 1-methyltetrahydro-β-carboline-3-carboxylic acid (MTCA) in an artificial gastric fluid [4,9]. A second impurity of the several contaminants in the tryptophan tablet associated with EMS was reported to be ''peak UV-5'' [12]. This compound was purified and the chemical structure was shown to be 3-phenylamino-L-alanine (PAA) [13,14]. PAA is chemically similar to 3-phenylamino-1,2-propanediol, an aniline derivative that has been isolated from samples of aniline denatured rapeseed oil [15].

The pathogenesis of EMS may be related to the possibility that MTCA and tetrahydro-β-carboline-3-carboxylic acid (TCCA) are considered to be putative precursors of the benzodiazepine receptor antagonist, β-carboline-3-carboxylate [16]. In addition, two diastereoisomers of MTCA were isolated from soy sauce as precursors of *N*-nitroso compounds showing direct-acting mutagenicity on *Salmonella typhimurium* TA 100 [17,18].

The aims of this paper are firstly to describe the methods for the separation, identification, and quantitative analyses of contaminants of L-tryptophan implicated in EMS, namely β-carbolines (norharman and harman), tetrahydro-β-carboline-3-carboxylic acids (TCCA and MTCA), EBT, and PAA. Secondly, the endogenous formation of tetrahydro-β-carboline-3-carboxylic acids in man and animals is examined. Finally the tissue distribution and metabolism of MTCA, EBT, and PAA in rats after acute and chronic administrations are described. The present paper covers six compounds as shown in Fig. 1.

2. Experimental

2.1. Materials

L-Tryptophan, norharman, harman, MTCA, *N*-phenylglycine, and *N*-(hydroxyphenyl) glycine were purchased from Sigma (St. Louis, MO, USA). 1-Ethyltetrahydro-β-carboline-3-carboxylic acid (ETCA) was prepared from L-tryptophan and propionaldehyde and heated to 240°C to yield 1-ethyl-β-carboline. TCCA was prepared from L-tryptophan and formaldehyde based on the procedure of Brossi et al. [19]. MTCA was a 12:1 diastereoisomeric mixture of (−)-(1S,3S)-MTCA and (−)-(1R,3S)-MTCA of known absolute configuration [20]. EBT was donated by Showa Denko, PAA was provided by Dr Uchiyama (NIHS Tokyo, Japan), 3-(phenylamino)lactic acid was synthesized from methyl acrylate [21], and propylsulfonic acid (PRS), benzensulfonic acid (SCX), and octyl-derivatized silica (C_8) bonded-phase columns were purchased from Varian (Harbor City, CA, USA).

2.2. Separation and quantitation

Samples for assays of β-carbolines, TCCA,

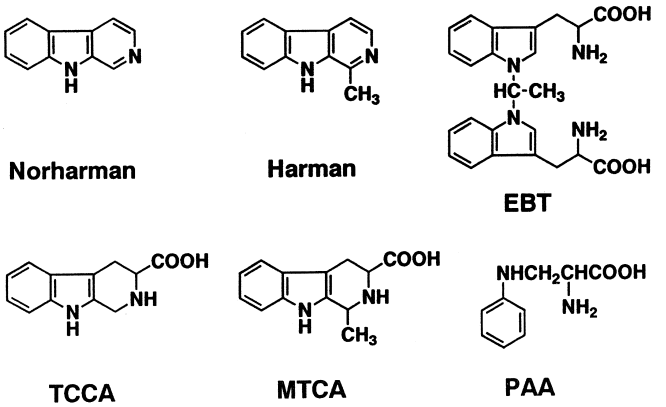

Fig. 1. The chemical structures of the main compounds described in this paper.

Table 1
Purification procedures using chemically-bonded material[a]

	β-Carboline	MTCA and PAA	EBT
Cartridge	PRS (3 ml)	SCX (3 ml)	C_8 (3 ml)
Conditioning	MeOH, 0.1 *M* HCl	MeOH, 0.1 *M* HCl	MeOH, Water
Apply	Sample (pH 1.5)	Sample (pH 1.5)	Sample (pH 9.3)
Wash	Water	0.1 *M* HCl, MeOH, water	Water, 10% MeOH
Rinse	Phosphate buffer (pH 9.1)	Phosphate buffer (pH 9.1)	
Elute	MeOH–0.2 N K_2HPO_4 (pH 8.8) (1:1)	MeOH–0.2 N K_2HPO_4 (pH 8.8) (1:1)	100% MeOH

[a] MTCA, 1-Methyltetrahydro-β-carboline-3-carboxylic acid; PAA, 3-(phenylamino)alanine; EBT, 1,1′-ethylidenebis(trypotphan).

MTCA, and PAA, were poured into a beaker containing the internal standards (I.S.) (1-ethyl-β-carboline for β-carbolines and ETCA for TCCA and MTCA) and were adjusted to pH 1.5 by the addition of 2 mol/l hydrochloric acid. An aliquot of 1 ml of carbonate buffer (0.005 *M*, pH 9.3) and 2 ml of distilled water were added to 1 ml of plasma or urine for the analysis of EBT. A purification procedure, involving a chemically-bonded material, is shown in Table 1 [22–26]. This extraction method is simpler and faster than liquid–liquid extraction [27] or silica gel thin-layer separation [17]. Our method required $<$30 min of preparation and gave excellent recoveries. The alkali or methanol eluate was injected directly onto the HPLC column, without evaporation, after passing through the single bonded silica column. For the quantitation of each compound, HPLC was performed [22–26]. The chromatographic conditions are shown in Table 2.

Fig. 2 (upper column) shows the HPLC chromatograms of TCCA, MTCA, and the I.S. in standard solution and beer. Fig. 2 (lower column) also shows chromatograms of norharman, harman, and the I.S. in standard solution and brandy. The HPLC system reported here gave a satisfactory separation of TCCA and two diastereoisomers of MTCA. Fig. 3 shows chromatograms of TCCA, MTCA in human urine. Fig. 4 shows chromatograms of EBT in plasma and urine of the rat administered EBT [24]. Fig. 5 shows a HPLC trace with fluorescence detection [excitation wavelength (ex.)=270 nm; emission wavelength (em.)=343 nm] (upper column) and UV detection

Table 2
HPLC conditions[a]

	β-Carboline	MTCA	EBT	PAA
Column	C_{18}-P, Nacalai tesque 150×4.6 mm I.D., 5 μm	ODS-80Tm, Tosoh, 250×4.6 mm I.D., 5 μm	ODS-5B, Showa Denko, 250×4.6 mm I.D., 5 μm	ODS-80Tm, Tosoh, 250×4.6 mm I.D., 5 μm
Mobile phase	32% MeOH, pH 3.0	32% MeOH, pH 3.0	23% Acetonitrile, pH 3.0	11% MeOH, pH 3.0
Flow-rate	0.6 ml/min	0.6 ml/min	0.6 ml/min	0.6 ml/min
Detector	FL 300/433 nm	FL 270/343 nm	UV 222 nm	ED Ag/AgCl 0.8 V, UV 222 nm, FL 270/343 nm
I.S.	1-Ethyl-β-carboline	ETCA	–	–

[a] MTCA, 1-Methyltetrahydro-β-carboline-3-carboxylic acid; EBT, 1,1′-ethylidenebis(tryptophan); PAA, 3-(phenylamino)alanine; ETCA, 1-ethyltetrahydro-β-carboline-3-carboxylic acid; FL, fluorescence; ED, electrochemical detection. Aqueous methanol was adjusted to pH 3.0 by phosphate buffer and phosphoric acid.

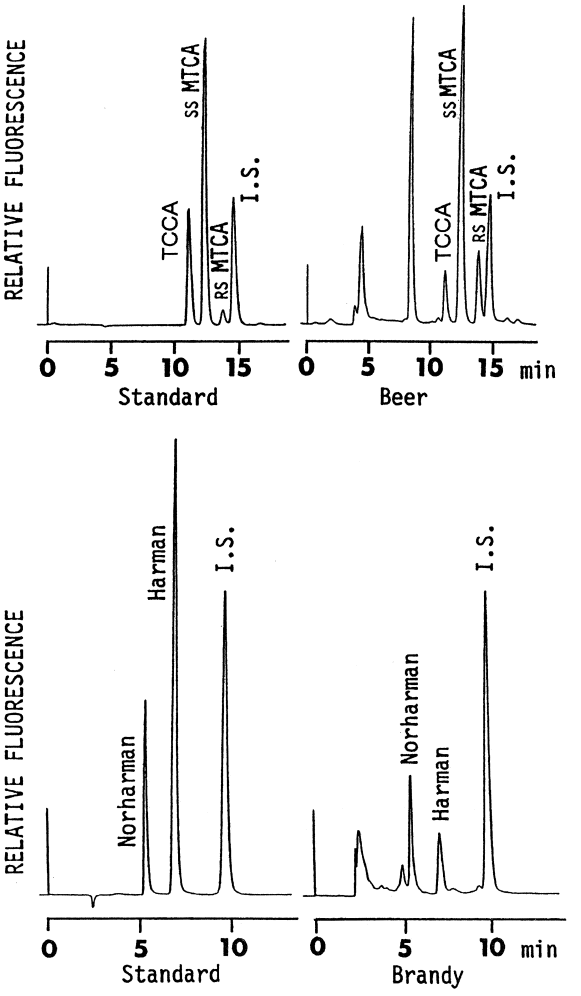

Fig. 2. High-performance liquid chromatograms of norharman and harman in a standard solution and brandy and TCCA and MTCA in a standard solution and beer.

(222 nm) (lower column) of PAA in the standard solution and rat brain administered PAA. The sensitivity for detecting PAA using electrochemical detection in the nanogram range [26] and was substantially greater than that obtained by employing fluorescence and UV monitoring (in the microgram range [25]).

2.3. Identification

2.3.1. β-Carbolines

LC–MS. A liquid chromatograph–tandem quadrupole mass spectrometer (LC–MS-QP1000; Shimadzu, Kyoto, Japan) equipped with a thermospray (TSP) interface (Vestec, Houston, USA) was used for the recording of mass spectra and selected ion monitoring (SIM). A C_{18}-P column was used for β-carbolines [22], whilst a ODS-80Tm was used for MTCA assay [23]. The mobile phase, consisting of 23% (v/v) aqueous methanol and 0.1 mol/l ammonium formate (pH 3.4), was delivered by a syringe pump (Shimadzu LC-6A) at a flow-rate of 1.0 ml/min. Samples were injected with a Rheodyne injector (Rheodyne 7125, Berkeley, CA, USA) fitted with a 100-μl loop. The vaporizer exit temperature was 140°C and ionization block temperature was 250°C. Positive ion thermospray mass spectra were obtained. Typical conditions for thermospray mass spectrometry were as follows: scan range=m/z 150–400 in 2 s, electron multiplier voltage=2450 V; preamplifier gain=7×10^7 V/A. The lower scan range limit of m/z (mass number) 150 was used to avoid any background interference from ammonium formate.

The thermospray ionization mass spectra of norharman and harman extracted from sake were identical with those obtained from standards. Formation of the MH^+ ion of either norharman and harman was observed as a base peak under thermospray ionization conditions. Mass chromatograms of norharman and harman obtained in the analyses of a standard mixture and an extract of sake are illustrated in Fig. 6. Peaks representing norharman as MH^+ were seen at m/z 169 at a retention time (t_R) of 5.8 min and from harman as MH^+ at m/z 183 at a t_R of 8.5 min. Hence LC–MS analysis provided a further structural identification.

The thermospray ionization mass spectra of TCCA and MTCA from the sake sample were identical to those obtained from standard samples. The mass spectra of the two diastereoisomers of MTCA were identical. Fig. 7 shows mass chromatograms of TCCA and MTCA in a standard solution and sake extract. Coinciding peaks originating from MH^+ of TCCA, (1S,3S)-MTCA, and (1R,3S)-MTCA are seen at m/z 217, 231, and 231, respectively, with retention times of 8.7, 10.7, and 12.7 min, respectively.

As β-carbolines and tetrahydro-β-carboline-3-carboxylic acids containing a carboxyl or amino group are not easily volatilized, direct analysis by GC–MS

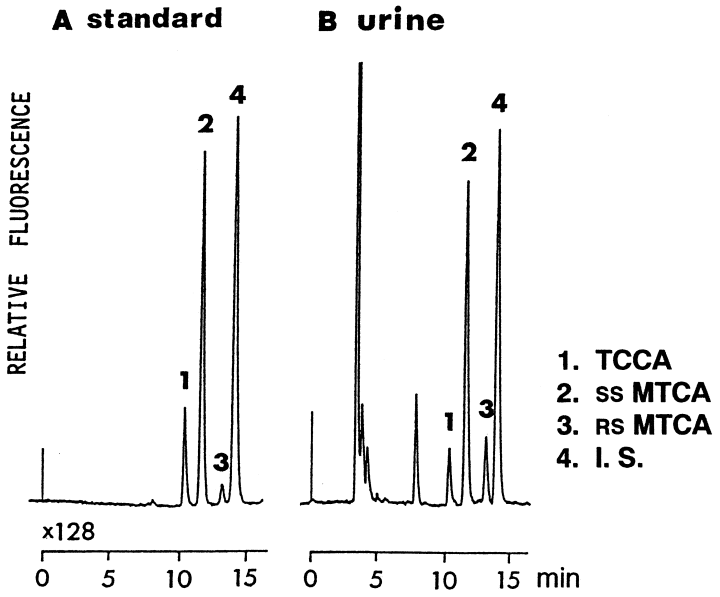

Fig. 3. Chromatograms of TCCA and MTCA in a standard mixture and human urine.

is difficult and derivatization reactions like esterification and amidation are necessary [27]. In our study [22,23], we succeeded in analyzing norharman, harman, TCCA, and MTCA directly and qualitatively with LC–MS.

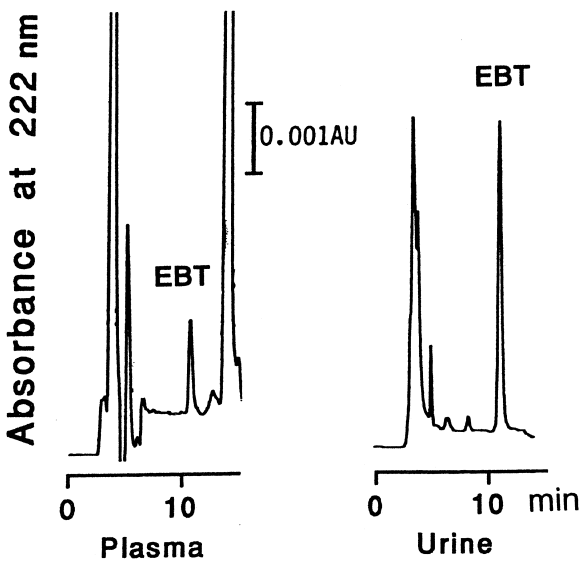

Fig. 4. Chromatograms of EBT in plasma and urine from an EBT-dosed rat.

2.3.2. Identification of PAA

For the analysis by GC–MS, a DX-300 GC–MS (JOEL, Tokyo, Japan), was connected on-line with a DA 5000 mass data analyzer (JOEL) [25]. Gas chromatographic separations were achieved with DB-17 fused-silica megabore column (30 m×0.53 mm I.D.) J & W Scientific, Folsom, CA, USA). The running conditions of the apparatus were as follows: carrier gas=helium; flow-rate=20 ml/min; column temperature=100°C programmed to increase to 200°C at a rate of 4°C/min; injection temperature=240°C; separation and insert temperature=245°C; ion-source temperature=240°C; ionization voltage=20 eV; ionization current=300 μA; acceleration energy=3.0 KV. The mass spectrometer apparatus was equipped with a multiple ion detector (MS-MID 06). Electron-impact mass spectra were obtained with scans time of 3 s over a mass range of 50–500 *m/z*.

3. Results and discussion

3.1. Contaminants in alcoholic beverages and foodstuffs

The foodstuff samples were purchased from local

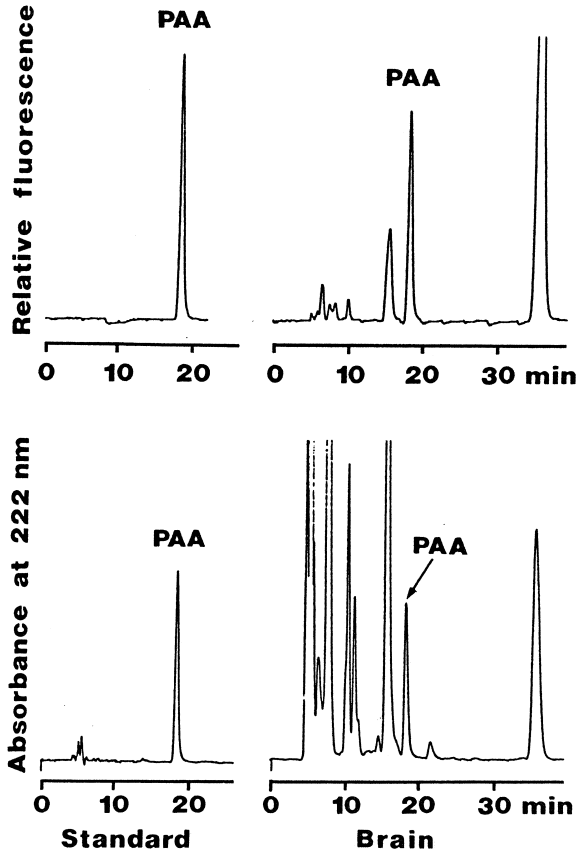

Fig. 5. Chromatograms of PAA in a standard solution and brain from a PAA-dosed rat.

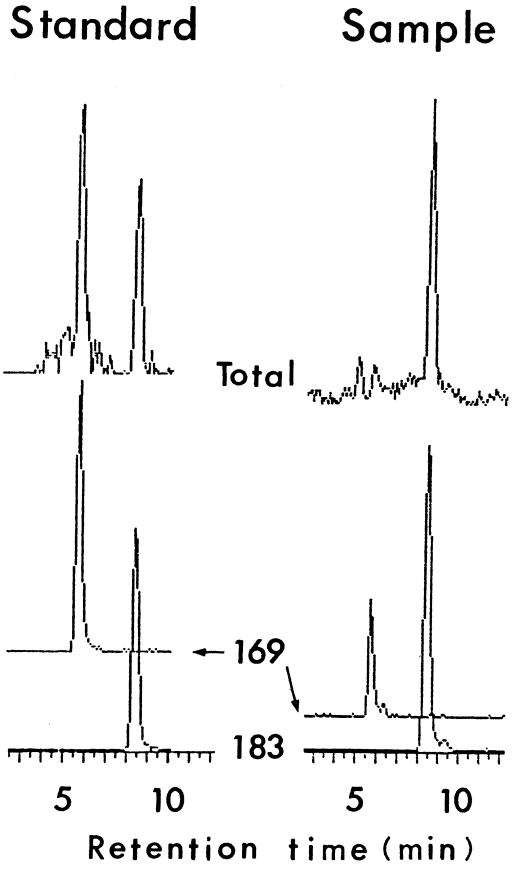

Fig. 6. Mass chromatograms of norharman (m/z 169) and harman (m/z 183) in a standard mixture and sake extract.

markets in Kobe, Japan. Aliquots of 1.0–10 ml of alcoholic beverages were diluted to 10–30 ml with distilled water and aliquots of 0.1–1 ml vinegar and soy sauce were diluted to 10 ml with water. Solid samples were homogenized, followed by centrifugation to extract the supernatant for analysis.

Table 3 gives the concentrations of β-carbolines and tetrahydro-β-carboline-3-carboxylic acids in various alcoholic beverages [22,23]. The diastereoisomeric mixture ratio of (1S,3S)-MTCA to (1R,3S)-MTCA was approximately 4:1, whereas a corresponding ratio of 12:1 was obtained in synthetic MTCA. The highest concentrations of both β-carboline and tetrahydro-β-carboline-3-carboxylic acids were obtained in Sake A. Sake and wine contained larger amounts of harman than beer and whisky. The concentration of MTCA was higher than that of TCCA in each alcoholic beverage. Bosin et al. [27] found that the concentration of TCCA in alcoholic beverages was about three to six times higher than MTCA. However, this was not consistent with our observations. Their chromatograms showed MTCA eluting at about 7 min after TCCA, with the peak height of MTCA in beer being higher than for TCCA. Judging from this point, beer seemed to contain a larger amount of MTCA than TCCA.

Table 4 shows the concentrations of β-carbolines and tetrahydro-β-carboline-3-carboxylic acids in fermented foodstuffs [22,23]. A great variability in concentration existed among the foodstuffs. In vinegar sample A, norharman, harman, TCCA, and MTCA were highest.

The presence of β-carboline and tetrahydro-β-carboline-3-carboxylic acids in plant-derived food-

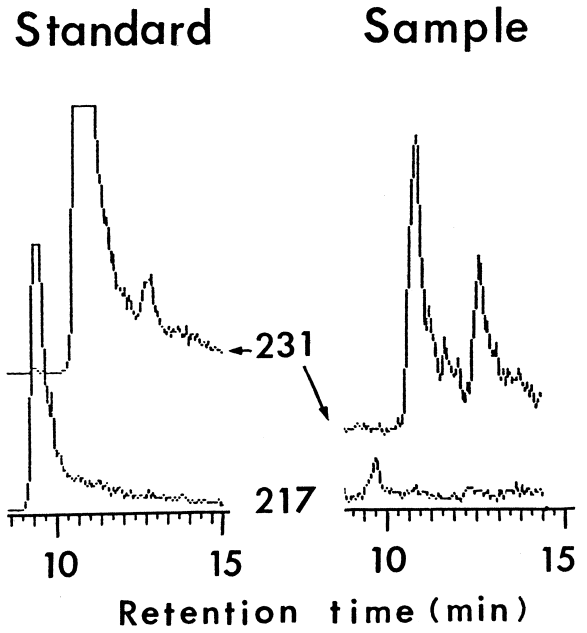

Fig. 7. Mass chromatograms of TCCA (m/z 217) and MTCA (m/z 231) in a standard solution and sake extract.

stuffs may indicate alkaloid formation through the Pictet-Spengler reaction of tryptophan with an aldehyde had occurred.

3.2. Excretion of tetrahydro-β-carboline-3-carboxylic acids

Urine was collected from 16 male and three female healthy volunteers and from nine healthy infants before weaning (age 0–3 months) [23]. Milk was collected from five mothers. Each urine sample was added to 6 mol/l hydrochloric acid (pH<2), and an aliquot of 1–8 ml was centrifuged to use the supernatant for subsequent analysis. Table 5 shows the concentrations of tetrahydro-β-carboline-3-carboxylic acids in breast milk and urine of human adults and infants. Adults excreted a significantly larger amount of MTCA than TCCA, whereas infants excreted comparable amounts of MTCA and TCCA.

The concentration of (1S,3S)-MTCA in human milk was significantly greater than TCCA. The concentration of total MTCA was similar to TCCA in infant urine. The average intake of formula milk was 110 g/day and the concentrations of TCCA and total MTCA in formula milk were 1.6 and 2.6 ng/g, respectively. On this basis, the intake per day of TCCA and of MTCA was calculated as 176 and 286 ng, respectively. The concentrations of TCCA and total MTCA in breast milk were 0.31 and 1.9 ng/ml, respectively. The average daily intake of breast milk was estimated to be 800 ml (750–850 ml) [28]. Accordingly, the intake of TCCA and of MTCA by breast-fed infants was about 240 and 1520 ng/day, respectively. In contrast, the concentrations of TCCA and total MTCA in infant urine were 4.9 and 4.0 ng/ml, respectively. Calculated urinary excretions of TCCA and MTCA were 2450 and 2000 ng/day, respectively, based on the assumption that infant urinary volume was 500 ml (350–550 ml) [29], which is greater than the calculated intake. Ingested tetrahydro-β-carboline-3-carboxylic acids in breast milk or formula milk are usually metabolized to some extent in the infant's body before excretion in urine. Thus, greater urinary excretion of tetrahydro-

Table 3
Concentrations of β-carbolines and tetrahydro-β-carboline-3-carboxylic acids in alcoholic beverages (mean±SD)[a]

	N	Alcohol (%, v/v)	Norharman (ng/ml)	Harman (ng/ml)	TCCA (ng/ml)	SS-MTCA (ng/ml)	RS-MTCA (ng/ml)
Sake	6	16	0.2±0.2	4.1±4.0	143±57	5277±2944	1581±871
Wine	5	14	0.5±0.2	8.5±14	167±98	3923±919	976±271
Beer	8	4.5–5.0	2.7±0.7	1.7±0.7	187±56	1032±517	260±135
Whisky	5	43	1.2±1.1	2.1±2.6	5±8	27±24	5±4
Shochu	2	25–43	0.1±0	0.1±0	2	28	6
Brandy	1	40	0.5	0.2	3	8	2
Sake A	1	16	67	590	1595	10 434	2294
Liqueur	1	14	26	85	1556	4109	1204

[a] TCCA, Tetrahydro-β-carboline-3-carboxylic acid; MTCA, 1-methyltetrahydro-β-carboline-3-carboxylic acid.

Table 4
Concentrations of β-carbolines and tetrahydro-β-carbolines in foodstuffs[a]

Products	Materials	Norharman (ng/ml or g)	Harman (ng/ml or g)	TCCA (μg/ml or g)	SS-MTCA (μg/ml or g)	RS-MTCA (μg/ml or g)
Vinegar						
A	Wheat	96	730	16	130	36
B	Rice	22	56	12	17	5
C	Grape	5.6	35	0.39	7.9	1.8
D	Corn	1.9	15	0.001	0.011	0.0001
Miso						
A	Soybean	8.2	35	2.6	104	26
B	Rice	15	0.9	1.1	5.2	0.89
C	Barley	45	9.6	2.6	66	16
Soy sauce						
A	Soybean	71	250	24	502	112
B	Soybean	15	130	5.9	97	19

[a] TCCA, Tetrahydro-β-carboline-3-carboxylic acid; MTCA, 1-methyltetrahydro-β-carboline-3-carboxylic acid.

β-carboline-3-carboxylic acids suggests that some tetrahydro-β-carboline-3-carboxylic acids were synthesized by intestinal bacteria or tissues of human infants.

3.3. Endogenous formation of MTCA in man

Male subjects were given 0.4 g/kg of ethanol, 0.5 g of tryptophan or water (control) [30]. Urinary excretions of TCCA and MTCA in healthy men are shown in Table 6. TCCA was markedly elevated at 1 h after tryptophan loading, being more than twice as high as the base value. MTCA excretion was increased at 1 h after ethanol intake with concomitant elevations in the MTCA to TCCA ratio.

3.4. Endogenous formation of TCCA and MTCA in rats

The distribution of TCCA and MTCA in various organs of rats was examined after a 17-h fast [31]. Rats ($N=72$) were divided into nine groups and were given the following drugs intragastrically: (a) control, saline; (b) L-tryptophan, as a single 160 mg/kg dose; (c) ethanol, a single 2 g/kg dose; (d) cyanamide–tryptophan, cyanamide i.p. at a dose of 20 mg/kg 2 h before 160 mg/kg of L-tryptophan; (e) cyanamide–tryptophan–ethanol, cyanamide 2 h before 160 mg/kg of tryptophan and 2 g/kg of ethanol. (b′) chronically tryptophan-treated rats, 160 mg/kg of L-tryptophan per day for 6 weeks. The other three

Table 5
Concentrations of tetrahydro-β-carboline-3-carboxylic acids in adult and infant urine and in breast milk (mean±SD)[a]

	N	TCCA (ng/ml)	SS-MTCA (ng/ml)	RS-MTCA (ng/ml)	Creatinine (mg/ml urine)
Urine					
Adult	19	114.0±78[b]	757.0±350[b]	171.0±96[b]	1.54±0.51[b]
Infant A[c]	4	5.1±1.4	3.2±1.5	0.4±0.3	0.09±0.06
Infant B	5	5.1±1.3	3.8±1.7	0.6±0.3	0.11±0.04
Breast milk	5	0.3±0.2[d]	1.6±0.7[d]	0.3±0.1	

[a] TCCA, Tetrahydro-β-carboline-3-carboxylic acid; MTCA, 1-methyltetrahydro-β-carboline-3-carboxylic acid.
[b] Significantly greater than the infant group, $P<0.05$.
[c] Infant A, formula-fed; infant B, breast milk-fed.
[d] Significantly less than the infant group, $P<0.05$.

Table 6
Excreted TCCA and MTCA in human urine after ethanol or tryptohan intake (mean±SD)[a]

Group	*N*	Before	1 h	2 h	3 h	4 h
TCCA (μg/h)						
Control	8	5.1±3.3	5.7±2.6	4.7±2.4	4.0±1.8	3.5±1.2
Tryptophan	7	2.4±1.2	5.8±2.9[b]	7.8±5.8[b]	5.6±4.1	4.5±3.4
Ethanol	7	2.4±1.1	2.7±1.5	1.2±0.5	1.3±0.6	1.2±0.7
MTCA (μg/h)						
Control	8	27.8±20.0	27.5±22.6	20.0±15.6	16.7±12.4	17.1±11.7
Tryptophan	7	20.4±12.0	32.7±26.8	30.9±29.5	23.4±19.3	21.9±19.3
Ethanol	7	26.7±4.1	40.5±14.2[b]	29.6±12.6	26.2±4.8	23.7±5.6

[a] TCCA, Tetrahydro-β-carboline-3-carboxylic acid; MTCA, 1-methyl-tyetrahydro-β-carboline-3-carboxylic acid.
[b] Significantly greater than its prior value, $P < 0.05$.

groups of rats were pair-fed nutritionally adequate liquid diets [32] purchased from the Oriental Yeast Co. (Japan) containing either ethanol (36% of total calories) or isocaloric carbohydrates as controls for 6 weeks before receiving the following drugs p.o. after an overnight fast: (a′) saline; (c′) 2 g/kg of ethanol; and (e′) 20 mg/kg of cyanamide i.p., 2 h before 160 mg/kg of L-tryptophan and 2 g/kg of ethanol. The name of each group coincided with that in Table 7.

One hour after dosing each rat was killed and the tissues were minced and homogenized with perchloric acid for subsequent analysis of the supernatants after centrifugation. The amount of TCCA excreted in the urine for 24 h (78.5 μg/24 h) was much higher than the amount of TCCA in rat chow (8.2 μg), revealing the endogenous formation of TCCA in rats. Table 7 shows extremely elevated levels of TCCA in kidney, blood, liver, and gastrocnemius muscle of rats after a single dose of L-tryptophan. These results reveal that in rats tryptophan is primarily metabolized to TCCA. This is not comparable with the results obtained from human urine (Table 6). However, in the groups that received chronic L-tryptophan supplementation (b′), no significant increases in TCCA levels were observed. Table 8 shows a significantly high level of MTCA in the kidney, blood, liver and muscle of rats treated acutely with cyanamide, tryptophan, and ethanol (group e). We did not analyze the L-tryptophan–ethanol combination study because treatment with ethanol alone did not affect TCCA and MTCA concentration in most rat tissues. Cyanamide

Table 7
TCCA concentrations in blood, various organs and gastrocnemius muscle (mean±SD)[a]

Group	No. of animals	TCCA (ng/g)			
		Blood	Kidney	Liver	Muscle
Acute					
(a) Control	11	1.2±0.3	3.1±0.8	0.6±0.1	2.5±0.9
(b) Trp	10	37±3.5[b]	363±46[b]	41±5.5[b]	7.5±0.8[b]
(c) EtOH	7	0.4±0.1	1.6±0.2	0.4±0.1	0.8±0.2
(d) Cy+Trp	6	1.5±0.3	6.2±1.1	2.0±0.3	0.9±0.2
(e) Cy+Trp+EtOH	11	1.4±0.7	4.9±1.1	0.9±0.2	1.9±0.5
Chronic					
(a′) Control	6	0.4±0.1	0.5±0.0	0.5±0.1	0.4±0.1
(b′) Trp	8	1.1±0.1	4.4±0.7	1.8±0.2	1.0±0.1
(c′) EtOH	4	0.9±0.7	1.0±0.6	0.2±0.0	0.5±0.3
(e′) Cy+Trp+EtOH	9	2.6±1.7	3.3±0.7	1.8±0.8	2.6±1.5

[a] TCCA, Tetrahydro-β-carboline-3-carboxylic acid; Trp, tryptophan; Cy, cyanamide.
[b] Significantly different from other groups, $P < 0.05$.

Table 8
MTCA concentrations in blood, various organs and gastrocnemius muscle (mean±SD)[a]

Group	No. of animals	MTCA (ng/g)			
		Blood	Kidney	Liver	Muscle
Acute					
(a) Control	11	0.6±0.1	0.7±0.1	0.6±0.1	1.3±0.2
(b) Trp	10	1.0±0.5	3.3±0.8	1.2±0.2	2.1±0.7
(c) EtOH	7	1.0±0.2	0.8±0.1	0.7±0.2	0.5±0.2
(d) Cy+Trp	6	1.3±0.1	1.7±0.3	1.4±0.1	1.0±0.0
(e) Cy+Trp+EtOH	11	27±2.8[b]	33±5.9[b]	11±1.0[b]	5.5±0.5[b]
Chronic					
(a′) Control	6	0.5±0.1	2.2±1.0	0.9±0.3	3.1±1.9
(b′) Trp	8	0.9±0.1	1.8±0.2	1.2±0.1	1.0±0.1
(c′) EtOH	4	1.2±0.1	1.1±0.4	0.8±0.1	1.0±0.2
(e′) Cy+Trp+EtOH	9	48±5.1[c]	104±13[c]	16.7±2.1[c]	7.4±0.8[c]

[a] MTCA, 1-methyltetrahydro-β-carboline-3-carboxxylic acid; Trp, tryptophan; Cy, cyanamide.
[b] Significantly different from other groups, $P < 0.05$.
[c] Significantly greater than the corresponding acute group, $P < 0.05$.

can alter aromatic amino acid decarboxylase activities, so that a pathway from tryptophan to 5-hydroxytryptamine is inhibited by cyanamide treatment and the condensation reaction of tryptophan with acetaldehyde may readily occur. A similar increase in the group e′, which had received the same treatment as the acute group after consuming a liquid diet containing ethanol for 6 weeks. Cyanamide-induced MTCA production is considered to be based on accumulation of acetaldehyde derived from ethanol because cyanamide can inhibit aldehyde dehydrogenase [33]. Accordingly acetaldehyde is demonstrated to be one of the factors affecting MTCA biosynthesis and one of the substrates which form MTCA in rat.

3.5. Tissue distribution between the rats administered MTCA, PAA, and EBT

3.5.1. Comparison of tissue distribution between the rats administered MTCA and PAA

After an overnight fast, rats were given by gastric gavage a single dose of MTCA (1.6 mg/kg; $N=5$) [34] or PAA (5 mg/kg; $N=5$) [26], respectively. They were sacrified at 5 h and their blood, brain, kidney and liver were dissected for analysis.

Table 9 shows levels of MTCA and PAA in blood and organ tissues [35]. MTCA was barely detectable in the brain, whereas very high levels of PAA were observed, indicating the passage of PAA through the blood–brain barrier. The concentration of PAA was

Table 9
Concentrations (ng/g tissue) at 5 h[a]

Group	Dose (mg/kg)	Concentration (ng/g)			
		Blood	Brain	Kidney	Liver
MTCA	1.6	3.8	0.2	75.4	12.9
PAA	5	899	2139	2225	1290
MTCA[b]	5	11.8	0.6	233	40
PAA/MTCA[b]		76	3500	10	32

[a] MTCA, 1-Methyltetrahydro-β-carboline-3-carboxylic acid; PAA, 3-(phenylamino) alanine.
[b] Calculated by dose.

highest in the kidney (2225 ng/g), and similar to that of brain (2139 ng/g) with a lower concentration in the liver (1290 ng/g). The lowest level of PAA was seen in the blood (899 ng/g), though this was 76 times higher than that of MTCA in blood as calculated by dose. The level of PAA in the liver was 32 times higher, and that in the kidney was ten times higher than the MTCA calculated by dose, respectively. Thus, as accumulation of PAA in each tissue was observed.

3.5.2. Comparison of excretion among the rats administered MTCA, EBT, and PAA

After overnight fast, rats were perorally administered the following drugs: (a) a single 1.6 mg/kg dose of MTCA ($N=15$) [34]; (b) a single 50 mg/kg dose of EBT ($N=15$) [24]; (c) a single 100 mg/kg dose of PAA ($N=12$) [25]. They were sacrificed at 5 h and blood and the contents of the large intestine were collected. Urine was collected separately in glass containers at the bottom of each cage for 5 h and 5–24 h.

Table 10 shows blood levels and excretions of PAA, MTCA, and EBT. The blood levels of PAA were 18 times higher than that of MTCA and 25 times higher than that of EBT. In addition, 35% of MTCA was excreted into urine and 61% of MTCA was found in the large intestine. Thirty-one percent of EBT was seen in the large intestine and 0.1% of EBT was excreted into urine, revealing a transfer of EBT to the large intestine without decomposition in the gastric fluid. In contrast, only 0.5% PAA was excreted into urine within 24 h and very little PAA remained in the large intestine. Thus, within 24 h most of the MTCA was excreted with or without absorption, and about 30% of EBT was excreted without absorption, whereas PAA was scarcely excreted. We suspect that the latter may be due to binding PAA in the circulation, such as with serum protein.

3.5.3. MTCA as a metabolite of EBT

After an overnight fast, rats were given by gastric gavage either saline (control) or a single 50 mg/kg dose of EBT ($N=14$) [24]. Urine was collected separately for 24 h. The blood, kidney, and liver were dissected at 1 h, whereas the large intestine was dissected from rats killed at 5 h. MTCA was shown to be excreted into the urine and was also located in the large intestine (Table 11). The total amount of MTCA in the large intestine in EBT-injected rats was about 16 times as large as the control group. In addition, significant accumulation of MTCA was shown in the blood, kidney, and liver at 1 h after dosing of EBT. These results suggest that MTCA is one of the decomposition products of EBT.

3.6. Effect of chronic treatment on MTCA and PAA concentrations in tissue

Twenty-nine rats were divided into four groups and orally administered the following drugs [26,34]: (1) a single 10 mg/kg dose of MTCA, (2) 10 mg/kg of MTCA for 6 weeks, (3) a single 5 mg/kg dose of PAA, (4) 5 mg/kg dose of PAA for 4 consecutive days at 5 h after the last dose, and (5) after an additional 72 h the rats were killed. In study (1), the highest concentration of MTCA at 1 h was found in

Table 10
Blood levels and excretions[a]

Group	Dose (mg/kg)	Blood 5 h (ng/ml)	Urine 0–24 h		Large intestine	
			μg	%	μg	%
MTCA	1.6	4	111	35.0	198	61
EBT	50	83	11	0.1	3136	31
PAA	100	4220	106	0.5	<1	0
MTCA[b]	100	2377	6950		12 375	
EBT[b]	100	166	22		6272	

[a] MTCA, 1-Methyltetrahydro-β-carboline-3-carboxylic acid; EBT, 1,1′-ethylidenebis(tryptophan); PAA, 3-(phenylamino)alanine.
[b] Calculated by dose.

Table 11
MTCA excretions and tissue concentrations in rats after EBT administration (mean±SD)[a]

Group	Urine 0–24 h (ng)	Large intestine 0–5 h (ng)	Blood 1 h (ng/g)	Kidney 1 h (ng/g)	Liver 1 h (ng/g)
Control	297	305	0.5±0.1	0.7±0.1	0.6±0.1
EBT 50 mg/kg	831	5097	4.7±1.3[b]	12.7±5.0[b]	7.0±4.0[b]

[a] MTCA, 1-Methyltetrahydro-β-carboline-3-carboxylic acid; EBT, 1,1′-ethylidenebis(trypophan).
[b] Significantly greater than the control group, $P < 0.05$.

the kidney (680 ng/g), followed by the liver, blood, and brain. In study (2), the concentration of MTCA in blood, brain and kidney after 6 weeks was approximately doubled by chronic treatment as shown in Table 12. In study (3), the concentration of PAA in the kidney (2225 ng/g) at 5 h was at the highest and was similar to that of the brain with a lower concentration seen in liver. The lowest level of PAA was seen in the blood (Table 12). The 4-day administration of PAA resulted in an approximately two-fold increase in tissue levels at 5 h after the last dose relative to that found following a single administration. The elevation found in blood was the greatest compared to other tissues. PAA tissue levels declined to trace concentrations within 72 h of discontinuing administration.

The data in Table 13 shows PAA tissue concentrations in twenty-six rats administered PAA in the diet (solid chow: 50 mg/kg) and killed at either 16 or 24 h ad libitum exposure to this diet [26]. A chronic feeding study of 6 weeks duration using solid PAA enriched diet was performed on 20 rats. Of these, 14 rats were killed at the end of 6 weeks and the remaining six rats were fed an identical

Table 12
Tissue concentrations (ng/g) in rats after acute loading and chronic administration of MTCA or PAA (mean±SD)[a]

Group	Dose (mg/kg)	Time (h)	*N*	Concentration (ng/g)			
				Blood	Brain	Kidney	Liver
MTCA							
Acute	10	1	6	76.6±15.3	4.1±0.7	680.0±187	363.0±66
Chronic	10	1	6	166.0±33[b]	7.2±1.0[b]	1300.0±134[b]	430.0±49[b]
PAA							
Acute	5	5	5	899.0±96	2139.0±147	2225.0±125	1290.0±57
Consecutive	5	5	6	2466.0±180[b]	3710.0±235[b]	4494.0±283	2857.0±106[b]
Consecutive	5	72	6	40.0±5	51.0±7	98.0±14	107.0±27

[a] MTCA, 1-Methyltetrahydro-β-carboline-3-carboxylic acid; PAA, 3-(phenylamino)alanine; Time, time since administration.
[b] Significantly greater than the corresponding acute value, $P < 0.05$.

Table 13
PAA concentrations in rats which received PAA as supplement in a solid diet (50 mg/kg chow)

Group	Concentration (ng/g±SEM)			
	Blood	Brain	Kidney	Liver
A[a]	146±17	598±180	1051±109	683±88
B	121±25	939±102	1094±60	659±46
C	1272±75[b]	1998±75[b]	2764±105[b]	1356±36[b]
D	10±2	26±3	68±11	84±9

[a] A, B, C, and D represent the groups divided by durations of administration. A, acute 16 h ($N = 3$); B, 24 h ($N = 3$); C, chronic 42 days ($N = 14$); D, 42+122 days ($N = 6$).
[b] Significantly greater than the corresponding acute value ($P < 0.05$).

control diet (no PAA) for an additional 12 days and then killed. In the acute studies, there were no significant increases in PAA content in any of the tissue at either the 16- or 24-h time points. A comparison of tissue levels following 42 days on a PAA diet (C) with those following 24 h exposure (B) showed a ten-fold increase in blood levels. Brain, liver and kidney levels increased approximately two-fold. At 12 days following the removal of PAA from the diet, drug levels decreased by 94–98%, detectable levels of the PAA were present in all tissues for at least 12 days following discontinuation.

3.7. Identification of PAA metabolites

Rats were given a single oral dose of 100 mg/kg PAA and urine was collected. The samples of trimethylsilyled (TMS) derivatives were analyzed by GC–MS [25].

Fig. 8 shows a total ion chromatogram (TIC) of TMS derivatives obtained from rat urine extract (upper column) and the mass spectra of those derivatives. The figure also shows the molecular ion chromatogram (*m*/*z* 223, 325, and 311) of each standard *N*-phenylglycine, 3-(phenylamino)lactic

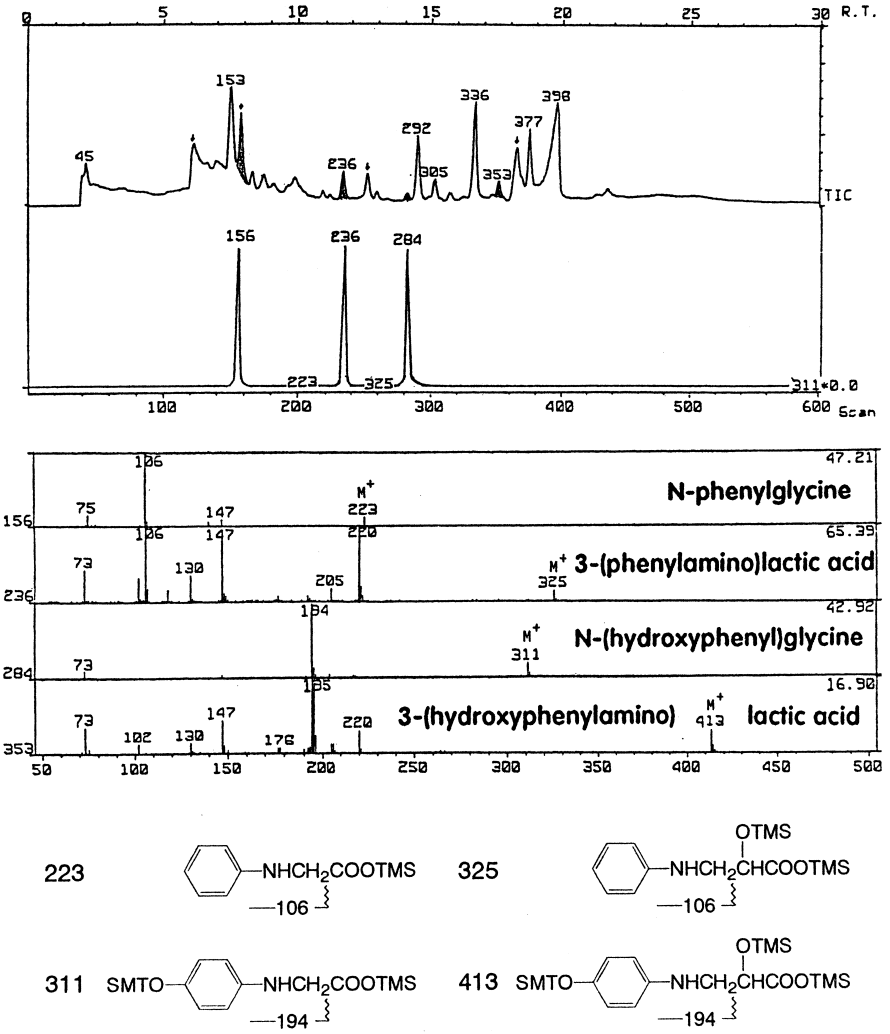

Fig. 8. Gas chromatograms of TMS derivatives in urine from rats administered with PAA and mass spectra of these derivatives.

acid, and *N*-(hydroxyphenyl)glycine (lower column). The ion peaks originating from TMS derivatives of standard *N*-phenylglycine, 3-(phenylamino)lactic acid, and *N*-(hydroxyphenyl)glycine were seen at t_R of 7.8, 11.8, and 14.2 min, respectively. The ion peak obtained from the urine extract appearing at a t_R of about 7.8 min had a molecular ion (m/z 223) and fragment ions similar to those for standard *N*-phenylglycine. Similarly, the ion peaks from the urine extract appearing at t_R values of about 11.8 and 14.2 min had molecular ions (m/z 325 and 311) and fragment ions similar to those for standard 3-(phenylamino)lactic acid and *N*-(hydroxyphenyl)glycine, respectively. Therefore, these peaks extracted from urine were identified from the similarity of their t_R and mass spectra compared with those of the standard specimen. The ion peak from the urine extract appearing at a t_R of 17.65 min had a molecular ion (m/z 413) and a base peak ion at m/z 194. *N*-phenylglycine and 3-(phenylamino)lactic acid had base peak at m/z 106, while *N*-(hydroxyphenyl)glycine had one at m/z 194, corresponding to the TMS derivative of the hydroxy group. Considering that the base peak (m/z 194) was the same as that of *N*-(hydroxyphenyl)glycine, the chemical structure of the ion peak was determined to be 3-(hydroxyphenylamino)lactic acid. Fragmentation patterns of these four compounds are shown under their mass spectra.

The pathway for the degradation of PAA could have similar features to that of phenylalanine or cystein conjugated with mercapturic acid derivatives [36]. The first step may be hydroxylation of PAA to hydroxy-PAA, after which a reaction may be catalyzed by phenylalanine hydroxylase (monooxygenase). The reductant may be tetrahydrobiopterine. The next step (a) may be the transamination of PAA (when the starting material was hydroxy-PAA, the product was written in parentheses) to 3-(phenylamino)pyruvic acid (hydroxyphenylaminopyruvic acid). The α-keto acid must be unstable, so it is immediately reduced by aldehyde reductase to 3-(phenylamino)lactic acid (hydroxyphenylaminolactic acid). The other step (b) may be decarboxylation by aromatic-L-amino acid decarboxylase to phenylethylenediamine (hydroxyphenylethylenediamine), which may subsequently be oxidized by monoamineoxidase to phenylaminoaldehyde (hydroxyphenylaminoaldehyde), immediately resulting in *N*-phenylglycine(hydroxyphenylglycine).

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