Beta-Carboline Alkaloids in Crude Drugs

Hironori TSUCHIYA,*,[a] Hiroshi SHIMIZU,[b] and Munekazu IINUMA[c]

Department of Dental Pharmacology, Asahi University School of Dentistry,[a] 1851 Hozumi, Hozumi-cho, Motosu-gun, Gifu 501–0296, Japan, Department of Manufacturing Pharmacy, Gifu Pharmaceutical University,[b] 5–6–1 Mitahora-higashi, Gifu 502–5858, Japan, and Gifu Prefectural Institute of Health and Environment,[c] 4–6–3 Noishiki, Gifu 500–8226, Japan.
Received October 28, 1998; accepted December 10, 1998

Beta-carboline alkaloids in crude drugs were quantified by a reversed-phase HPLC method without interference from their artifactual formation during analysis and with fluorometric detection specific to each individual analyte. 1-Methyl-β-carboline, β-carboline, 7-hydroxy-1-methyl-β-carboline, 7-methoxy-1-methyl-β-carboline, 1-methyl-1,2,3,4-tetrahydro-β-carboline and 1,2,3,4-tetrahydro-β-carboline showed a wide distribution in the crude drugs and the former two β-carbolines were detected in all those tested. Schisandrae Fructus, Pinelliae Tuber, Evodiae Fructus and *Passiflora incarnata* contained relatively large amounts of β-carbolines at ng—μg/g dry weight levels. Beta-carboline alkaloids may be responsible for the pharmacological effects of crude drugs as the potent active substances.

Key words β-carboline alkaloids; crude drugs; HPLC analysis

A series of β-carbolines with the 9*H*-pyrido[3,4-*b*]indole structure have been considered as pharmacologically active mammalian alkaloids since findings of their presence in humans and their various biological activities.[1,2] Beta-carbolines are produced by a condensation reaction between indoleamines (tryptamine, serotonin and/or tryptophan) and carbonyl compounds (aldehydes and/or α-keto acids).[3] Although there is a possibility that β-carbolines are endogenously produced (partly biosynthesized) in humans,[1,3] their overwhelmingly large amounts are more likely to be supplied exogenously.[4] Beta-carbolines are originally alkaloidal components in plants,[1,5] therefore they would be present in crude drugs as detected in limited kinds of medicinal plants recently.[6,7] If crude drugs contain significant amounts of β-carbolines, such alkaloids are speculated to participate in their pharmacological effects.

The representative β-carboline alkaloids originating from plants are 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTBC) and 1,2,3,4-tetrahydro-β-carboline (TBC) which are oxidized to 1-methyl-β-carboline (MBC) and β-carboline (BC), respectively. MBC is also structurally modified to 7-hydroxy-1-methyl-β-carboline (7-OH-MBC) and 7-methoxy-1-methyl-β-carboline (7-M-MBC). Since the condensation reaction for β-carbolines occurs under experimental conditions to form β-carbolines as the artifacts,[8] the analytical method without interference from such an artifactual formation is essential to determine the original content of β-carbolines in crude drugs. The artifactual formation was successfully suppressed by treating samples with fluorescamine prior to HPLC analysis.[9,10] In the present study, that method was applied to crude drugs for the compositional assessment of β-carboline alkaloids as the potent active substances.

Materials and Methods

Chemicals MTBC, TBC and 2-ethyl-1,2,3,4-tetrahydro-β-carboline (ETBC) were synthesized by the method of Tsuchiya *et al.*[11] MBC, BC, 7-OH-MBC, 7-M-MBC and 3-hydroxymethyl-β-carboline (HMBC) were purchased from Funakoshi (Tokyo, Japan). Fluorescamine was obtained from Fluka (Buchs, Switzerland). Acetonitrile of HPLC grade (Kishida, Osaka, Japan) was used for preparing the mobile phase and fluorescamine solution. All other reagents were of the highest grade available. Water was redistilled using an all-glass apparatus after purifying by a Milli-RO water purification system (Nihon Millipore, Tokyo, Japan).

Sample Preparation All the dried crude drugs approved by Japanese Pharmacopoeia XIII[12] were purchased from Nakarai Koshindo (Kobe, Japan) on April 15, 1997. *Passiflora incarnata* and *Tribulus terrestris* were obtained from Matsuura Yakugyo (Nagoya, Japan) on May 16, 1997 and June 6, 1997, respectively. The pulverized samples were homogenized in 0.1 M HCl containing 10 mM semicarbazide and the homogenates were centrifuged at 10000×*g* for 15 min. The supernatants were diluted with 0.1 M HCl containing 10 mM semicarbazide as required (0.5—50 mg/ml) according to the alkaloid concentrations. To 0.5 ml of each diluent, 50 μl of an aqueous solution of ETBC (200.0 ng/ml) and HMBC (25.0 ng/ml) was added. The mixture was vortex-mixed with 0.5 ml of 2 M potassium phosphate buffer (pH 8.5) and 0.5 ml of a fluorescamine solution in acetonitrile (5 mg/ml) for 30 s, and then with 0.5 ml of 2 M potassium phosphate buffer (pH 8.5) containing glycine (100 mg/ml) for 30 s, followed by three-step extractions.[9,10] The finally obtained extract was evaporated to dryness and the residue was dissolved in 200 μl of 0.2% (v/v) trifluoroacetic acid solution. An aliquot (50—100 μl) of the resulting solution was loaded onto an HPLC column.

HPLC Analysis The HPLC system consisted of an LC-10AD liquid chromatograph (Shimadzu, Kyoto, Japan) connected to an SCL-10A system controller (Shimadzu) and a DGU-4A degasser (Shimadzu), a 7125 sample injector (Rheodyne, Cotaci, U.S.A.) and a Shim-pack CLC-C8 (M) column (25 cm×4.6 mm i.d., particle size of 5 μm, Shimadzu) placed in a CTO-6A column oven (Shimadzu). The separation was performed by delivering the mobile phase, acetonitrile/trifluoroacetic acid/water (18.0 : 0.2 : 81.8, v/v/v), at a flow rate of 1.0 ml/min and at a column temperature of 50 °C. Based on spectral measurement and retention time of each analyte,[7,13] 7-OH-MBC, HMBC, BC, MBC and 7-M-MBC were detected by an RF-550 spectrofluorometric detector (Shimadzu) which was time-programmed as follows: excitation/emission wavelengths (nm) were 325/417 at 0—7.50 min for 7-OH-MBC, 300/447 at 7.50—10.00 min for HMBC and BC, 300/430 at 10.00—14.50 min for MBC and 330/417 at 14.50—18.00 min for 7-M-MBC. An RF-535 fluorometric detector (Shimadzu) was connected in series to detect MTBC, TBC and ETBC at 275 nm for excitation and 350 nm for emission. Beta-carbolines in oxidized- and reduced-form were quantified based on the peak area ratios to HMBC and ETBC, respectively. Their contents in crude drugs were corrected by the recoveries obtained from spiking experiments and the dilution factors.

Analytical Evaluation To evaluate the recovery and analytical precision, a mixture of 7-OH-MBC (0.4—4.0 ng/ml), BC (0.2—2.0 ng/ml), MBC (0.2—2.0 ng/ml), 7-M-MBC (0.4—4.0 ng/ml), MTBC (2.0—20.0 ng/ml) and TBC (2.0—20.0 ng/ml) was added to crude drug diluent samples. Replicate spiked samples were analyzed on the same day ($n=6$ for recovery and intra-assay precision) and on different days ($n=4$ for inter-assay precision) as described above.

Results and Discussion

All analytes including two internal standards were simultaneously analyzed by a single run of HPLC with fluorometric

* To whom correspondence should be addressed.

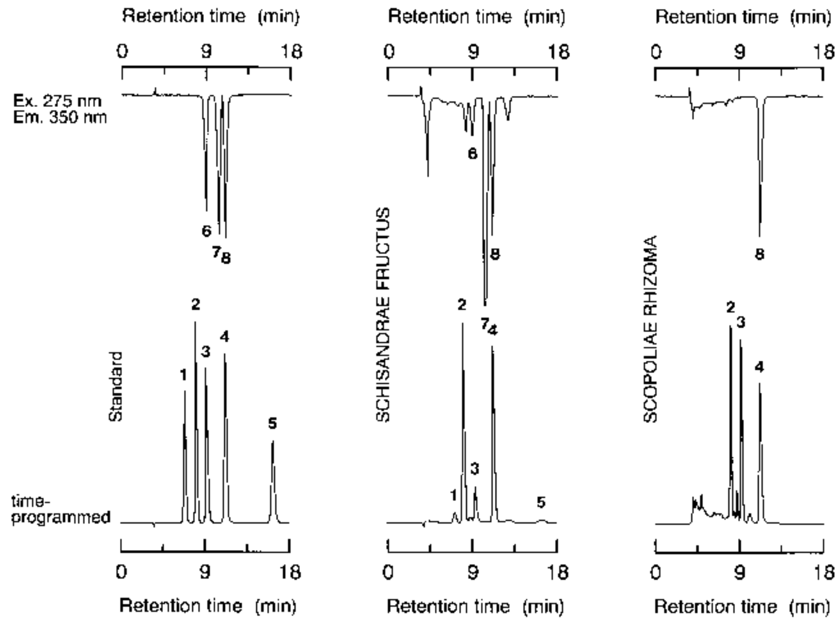

Fig. 1. High-Performance Liquid Chromatograms Obtained from Standard and Crude Drug Samples

Standard: 7-OH-MBC (3.0 ng/ml), BC (1.5 ng/ml), MBC (1.5 ng/ml), 7-M-MBC (3.0 ng/ml), TBC (15.0 ng/ml) and MTBC (15.0 ng/ml). Crude drugs: homogenates of Schisandrae Fructus (10 mg/ml) and Scopoliae Rhizoma (10 mg/ml). Peaks: 1=7-OH-MBC, 2=HMBC (internal standard), 3=BC, 4=MBC, 5=7-M-MBC, 6=TBC, 7=MTBC and 8=ETBC (internal standard).

Table 1. Recovery and Analytical Precision

Crude drug	Recovery, intra-assay CV and inter-assay CV (%)																	
	7-OH-MBC			BC			MBC			7-M-MBC			TBC			MTBC		
Schisandrae Fructus	88.0	1.3	2.5	95.3	3.7	4.1	99.3	5.5	5.7	94.7	0.7	1.6	115.9	3.8	7.0	98.1	4.4	6.7
Pinelliae Tuber	92.3	1.0	1.4	88.0	1.2	1.7	87.3	0.9	1.5	90.7	1.0	1.5	96.4	5.4	7.2	100.1	1.8	2.3
Scopoliae Rhizoma	92.3	2.2	3.5	80.0	1.0	1.4	82.7	1.1	3.3	92.0	2.9	3.2	99.3	1.3	3.2	100.8	1.1	2.7
Ophiopogonis Tuber	83.3	5.6	6.5	88.0	5.2	6.4	97.3	6.1	6.4	80.7	5.4	5.6	99.7	4.2	5.1	93.6	2.1	2.4
Ginseng Radix	80.3	8.7	6.6	68.0	7.8	9.0	78.7	5.4	7.3	66.0	3.6	6.3	118.8	2.2	3.5	99.9	2.5	3.5
Bupleuri Radix	85.7	5.4	8.4	101.3	6.8	10.1	95.3	6.8	9.8	95.0	6.3	9.8	100.5	6.2	6.8	110.1	1.7	3.4
Puerariae Radix	98.0	5.5	6.3	79.3	3.7	4.5	86.7	3.6	4.3	91.0	8.4	8.7	100.9	1.8	4.5	99.7	4.3	6.1
Polygalae Radix	81.7	3.0	2.9	94.7	5.2	7.0	95.3	4.4	5.8	88.3	0.7	2.8	100.2	2.3	6.5	98.3	2.2	2.9
Gentianae Radix	99.0	1.3	1.4	97.3	1.6	2.4	98.0	1.7	3.2	101.3	0.4	0.4	98.5	4.1	4.7	98.4	5.0	6.1
Arecae Semen	98.3	0.6	1.6	90.7	1.5	2.2	91.3	0.7	0.8	95.0	0.2	0.6	101.9	1.5	4.9	104.1	3.6	6.4
Evodiae Fructus	95.7	2.0	4.9	104.7	2.6	4.4	89.3	5.1	8.4	102.3	1.4	2.3	—	—	—	—	—	—
Passiflora incarnata	92.3	9.2	7.9	101.3	10.6	13.9	92.0	10.7	10.9	94.0	10.2	9.3	90.4	4.1	5.5	102.5	7.0	8.3
Tribulus terrestris	92.7	2.3	2.0	72.0	2.0	1.6	73.3	4.2	4.0	89.0	1.0	0.9	108.5	4.3	4.8	100.5	5.1	6.6

Recovery and precision were evaluated by analyzing replicate crude drug samples spiked with standards of 0.2—20.0 ng/ml (n=6 for recovery, n=6 for intra-assay CV and n=4 for inter-assay CV). —, not determined.

detection specific to each β-carboline as shown by the representative chromatograms (Fig. 1). The obtained peaks reflect the original contents in crude drugs, not including the artifacts because occurrence of the condensation reaction during analysis was completely suppressed by the fluorescamine-pretreatment, in which the samples were reacted with fluorescamine in the first step of an extraction procedure to remove the precursor indoleamines from the analytical system and only the purified original analytes were subjected to HPLC separation.[9,10)]

Both recovery and reproducibility were so high that the present method was applicable to the quantitation of β-carbolines in crude drugs as shown by the typical results (Table 1). More than 80% of the spiked β-carbolines were recovered from the homogenate samples, and intra- and inter-assay CVs were within 8% except for a part of the samples. The quantitative range was 0.01—20.0 ng/ml for MBC and BC, 0.02—20.0 ng/ml for 7-OH-MBC, 0.03—20.0 ng/ml for 7-M-MBC and 0.1—50.0 ng/ml for MTBC and TBC.

Quantitative analyses revealed that β-carboline alkaloids were contained in various crude drugs (Table 2). BC and MBC were detected in all those tested. 7-OH-MBC showed relatively wide distribution. However, the presence and content of MTBC, TBC and 7-M-MBC depended on the kind of crude drugs analyzed. Some β-carbolines were previously detected in Rutaceae, Leguminosae, Passifloraceae, Loganiaceae, Plamae, *etc.*[5)] MBC, BC, MTBC and/or TBC were also found in plants belonging to Solanaceae and their products such as tomato, tomato juice, tabasco and tobacco.[4,10,13)] The present quantitation has proven that the crude drugs cor-

Table 2. Beta-Carboline Alkaloids in Crude Drugs

Crude drug	Content (ng/g dry weight)					
	7-OH-MBC	BC	MBC	7-M-MBC	TBC	MTBC
Schisandrae Fructus	125±91	28.7±0.8	114±14	3.2±2.6	513±40	$7.17\pm0.07\times10^3$
Pinelliae Tuber	ND	79.1±13.8	82.0±17.4	11.2±2.8	61.9±50.5	$7.84\pm0.28\times10^3$
Scopoliae Rhizoma	10.3±5.9	170±1	132±1	ND	42.3±34.5	ND
Evodiae Fructus	400±75	$8.24\pm0.13\times10^3$	636±134	ND	ND	ND
Ophiopogonis Tuber	2.4±1.2	27.0±1.7	46.6±4.3	ND	1.6±1.4	4.4±3.8
Ginseng Radix	ND	29.9±3.8	65.0±11.2	ND	ND	ND
Bupleuri Radix	32.7±0.8	122±4	56.9±2.1	ND	57.0±1.3	ND
Puerariae Radix	ND	229±24	96.8±29.1	ND	ND	ND
Polygalae Radix	127±45	90.8±5.5	118±5.6	7.7±6.7	ND	ND
Gentianae Radix	9.1±3.2	71.5±2.6	9.9±0.9	ND	ND	ND
Arecae Semen	ND	21.6±0.5	11.6±0.2	ND	12.6±1.1	33.8±0.4
Zingiberis Rhizoma	172±11	129±11	190±15	11.9±1.4	ND	ND
Caryophylli Flos	ND	95.0±3.4	56.2±2.3	ND	ND	ND
Forsythiae Fructus	30.2±1.1	9.3±0.3	4.2±0.1	1.4±0.1	33.0±5.3	11.3±8.0
Plantaginis Semen	ND	15.4±0.4	9.3±0.2	ND	ND	ND
Passiflora incarnata	410±201	68.3±5.3	126±27	30.8±16.9	81.1±66.2	ND
Tribulus terrestris	223±112	131±11	99.5±13.2	ND	ND	ND

Each value represents mean±S.E. (n=3—5). ND, not detected.

responding to that classification uniformly contain β-carboline alkaloids. In particular, Schisandrae Fructus and Pinelliae Tuber contained MTBC at μg/g dry weight levels. Evodiae Fructus was superior in content of BC, MBC and 7-OH-MBC to the other crude drugs. The relatively large amounts of β-carbolines in oxidized-form were present in Scopoliae Rhizoma, Puerariae Radix, Polygalae Radix, Zingiberis Rhizoma, Bupleuri Radix, *Passiflora incarnata* and *Tribulus terrestris*.

Beta-carbolines have a variety of neuropharmacological activities.[2,3,14] They include benzodiazepine antagonism (IC_{50} for inhibition of flunitrazepam binding: 5—7 μM for MBC, 6—8 μM for BC, 64 μM for 7-OH-MBC, 920 μM for TBC and 1450 μM for MTBC),[15,16] inhibition of monoamine oxidase (IC_{50} for inhibition of calf liver and mouse brain monoamine oxidase: 0.75 nM—20 μM for BC, 5 nM—3.3 μM for MBC, 15—80 nM for 7-M-MBC, 27.5 nM—5.8 μM for 7-OH-MBC, 42.5 nM—120 μM for MTBC and 58 μM for TBC),[17,18] and inhibition of biogenic amine uptake (IC_{50} for inhibition of serotonin, dopamine and epinephrine uptake: 1.0—6.4 μM for MTBC, 3.0—6.2 μM for TBC and 3.2 μM for 7-M-MBC).[3] Since Schisandrae Fructus and Pinelliae Tuber influence the central nervous system, their major component, MTBC may act as a neuroactive substance. Evodiae Fructus, *Passiflora incarnata* and *Tribulus terrestris* have been used as a sedative agent, which may be attributed to BC, MBC, 7-OH-MBC and 7-M-MBC.[6] While β-carbolines have the affinity for benzodiazepine receptors, both peripheral- and central-type benzodiazepine receptors exist in parotid and submandibular glands of rats.[19,20] Rat xerostomia induced by diazepam was restored by Byakkoka-ninjin-to (45—90 mg/kg, *p.o.*) and Bakumondo-to (90 mg/kg, *p.o.*),[21] which contain Pinelliae Tuber, Ginseng Radix and Ophiopogonis Tuber. As an active substance in these crude drugs, β-carbolines may promote salivary secretion by binding to benzodiazepine receptors in salivary glands. Several β-carbolines also show antimicrobial activity against various bacteria and fungi, and their minimal inhibitory concentrations range 0.9—15 μg/disc for BC, 1.9—120 μg/disc for MBC, 100—>500 μg/ml for 7-OH-MBC and 50—>500 μg/ml for 7-M-MBC.[22,23] Therefore, the β-carbolines detected in certain crude drugs traditionally used for intestinal diseases potentially contribute to their therapeutic effects by influencing intestinal microflora. Beta-carboline alkaloids have different activities in addition to their neuroactive and antimicrobial effects,[2,3,14] suggesting their potent pharmacological significance in crude drugs.

Acknowledgments This study was partly supported by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science, Sports and Culture of Japan.

References

1) Airaksinen M. M., Kari I., *Med. Biol.*, **59**, 21—34 (1981).
2) Rommelspacher H., May T., Susilo R., *Planta Med.*, **57** (S1), S85—S92 (1991).
3) Melchior C., Collins M. A., *CRC Crit. Rev. Toxicol.*, **9**, 313—356 (1982).
4) Tsuchiya H., Yamada K., Todoriki H., Hayashi T., *J. Nutr. Biochem.*, **7**, 237—242 (1996). Erratum: **7**, 414—415 (1996).
5) Allen J. R. F., Holmstedt B. R., *Phytochem.*, **19**, 1573—1582 (1980).
6) Rehwald A., Sticher O., Meier B., *Phytochem. Anal.*, **6**, 96—100 (1995).
7) Tsuchiya H., Hayashi H., Sato M., Shimizu H., Iinuma M., *Phytochem. Anal.*, in press.
8) Bosin T. R., Holmstedt G., Lundman A., Beck O., *Anal. Biochem.*, **128**, 287—293 (1983).
9) Tsuchiya H., Ohtani S., Yamada K., Takagi N., Hayashi T., *J. Pharm. Biomed. Anal.*, **12**, 1547—1553 (1994).
10) Tsuchiya H., Yamada K., Kato H., Hayashi H., Miyazaki T., Hayashi T., *Phytochem. Anal.*, **6**, 297—301 (1995).
11) Tsuchiya H., Ohtani S., Yamada K., Takagi N., Todoriki H., Hayashi T., *J. Pharm. Sci.*, **83**, 415—418 (1994).
12) The Ministry of Health and Welfare of Japan, Japanese Pharmacopoeia 13th Ed., Hirokawa, Tokyo, 1996.
13) Tsuchiya H., Sato M., Hayashi H., Kato H., Kureshiro H., Hayashi T., *Chromatographia*, **43**, 419—425 (1996).
14) Airaksinen M. M., Kari I., *Med. Biol.*, **59**, 190—211 (1981).
15) Airaksinen M. M., Mikkonen E., *Med. Biol.*, **58**, 341—344 (1980).
16) Rommelspacher H., Nanz C., Borbe H. O., Fehske K. J., Müller W. E., Wollert U., *Eur. J. Pharmacol.*, **70**, 409—416 (1981).
17) McIsaac W. M., Estevez V., *Biochem. Pharmacol.*, **15**, 1625—1627 (1966).
18) Buckholtz N. S., Boggan W. O., *Biochem. Pharmacol.*, **26**, 1991—1996 (1977).
19) De Souza E. B., Anholt R. R. H., Murphy K. M. M., Snyder S. H.,

Kuhar M. J., *Endocrinol*., **116**, 567—573 (1985).
20) Yamagishi H., Kawaguchi M., *Biochem. Pharmacol.*, **55**, 209—214 (1998).
21) Kawaguchi M., Ohkubo M., Yamagishi H., Abstracts of Papers, The 36th Annual Meeting of Japanese Association for Oral Biology, Osaka, October 1994, p. 155.
22) Prinsep M. R., Blunt J. W., Munro M. H. G., *J. Nat. Prod*., **54**, 1068—1076 (1991).
23) Ahmad A., Khan K. A., Sultana S., Siddiqui B. S., Begum S., Faizi S., Siddiqui S., *J. Ethnopharmacol*., **35**, 289—294 (1992).