Investigation of "Signal" Constituents for the Evaluation of Animal Crude Drugs. I.[1] Free Amino Acids and Total Amino Acids

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The contents of free amino acids (FAA) and total amino acids (TAA; including peptide and protein forms) in 10 animal drugs were determined using precolumn-derivatization high performance liquid chromatography (HPLC). The results showed that the FAA and TAA content was characteristic for a particular animal crude drug. For example, Bezoar Bovis contained much more taurine relative to the FAA and TAA content compared with other crude drugs, and kokurozin contained much more TAA and much less secondary amino acids relative to TAA compared with koukuzin. FAA and TAA were proved to be "signal" constituents for the evaluation of animal crude drugs.

Keywords animal crude drug; amino acid; orthophthalaldehyde; 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; precolumn derivatization HPLC

We are investigating analytical methods for the evaluation of animal crude drugs which have been used as various Chinese medicines and are looking for "signal" characteristic constituents for the evaluation. This is because most of them have not been included in the monographs of both the Pharmacopoeia of Japan (JP) and the Japanese Standards of Pharmaceutical Ingredients (JSPI). Even Cervi Parvum Cornu, one of the most popular animal crude drugs, is not included in JP and JSPI. As part of our study of the analysis of constituents of crude drugs, research into characteristic "signal" constituents was carried out to help in the evaluation of animal crude drugs. We presumed that the contents and constituents of the free amino acids (FAA) and total amino acids (TAA; including peptide and protein forms) in every crude drug would be characteristic. Determination of amino acids was carried out by precolumn-derivatization high performance liquid chromatography (HPLC)[2] applied to 10 kinds of animal crude drugs: Bezoar Bovis and Ostreae Testa in JP, Asini Gelatinum, Lumbricus, Cicadae Periostracum and Amydae Carapax in JSPI, and Cervi Parvum Cornu, Hippocampus, Kokurozin and Koukuzin which were readily available.

Experimental

Samples Bezoar Bovis, Ostreae Testa, Lumbricus, Cicadae Periostracum and Amydae Carapax are available commercially on the Japanese Market. Bezoar Bovis consisted of three sample from Australia, North America and Brazil obtained from Nihon Funmatu Yakuhin Co., Ltd. (Osaka, Japan) which met the requirements of JP. Ostreae Testa consisted of three samples which met the requirements of JP, one was from Japan and obtained from Nakajima Shyoyaku Co., Ltd. (Kyoto, Japan) and the others were unknown location and obtained from Koshiro Seiyaku Co., Ltd. (Osaka, Japan). Lumbricus consisted of three samples from Thailand and obtained from Nihon Funmatu Yakuhin Co., Ltd., Cicadae Periostracum consisted of three samples from China and obtained from Takasago Yakugyo Co., Ltd. (Osaka, Japan), Tochimoto Tenkaido Co., Ltd. (Osaka, Japan) and Kinokuniya Kanyakkyoku Co.,

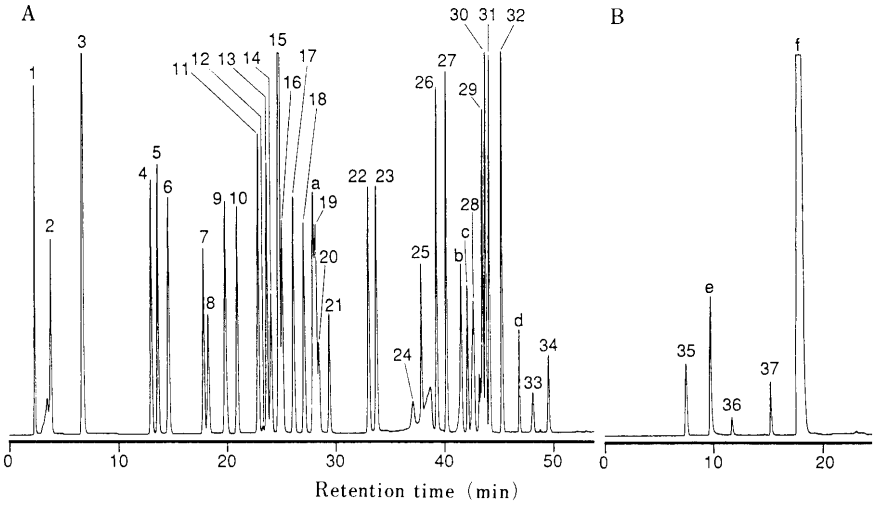

Fig. 1. Typical Chromatograms of Primary (A) and Secondary (B) Amino Acids Obtained from Standard Solutions

Amino acid concentrations of standard solution (μmol/ml) 1: P-Ser; 0.1, 2: Asp; 0.1, 3: Glu; 0.1, 4: α-AAA; 0.1, 5: Ser; 0.1, 6: Asn; 0.1, 7: Gln; 0.1, 8: PEA; 0.1, 9: Gly; 0.1, 10: Thr; 0.1, 11: Cit; 0.1, 12: Cys-Cys; 0.1, 13: 1Mehis; 0.1, 14: 3Mehis; 0.1, 15: Arg; 0.2, 16: β-Ala; 0.1, 17: Ala; 0.1, 18: Tau; 0.1, 19: Car; 0.1, 20: Ans; 0.1, 21: Hcy-Hcy; 0.1, 22: Tyr; 0.1, 23: α-ABA; 0.1, 24: His; 0.1, 25: Ala-Hcy; 0.1, 26: Val; 0.1, 27: Met; 0.1, 28: Hylys; 0.1, 29: Trp; 0.1, 30: Ile; 0.1, 31: Phe; 0.1, 32: Leu; 0.1, 33: Orn; 0.1, 34: Lys; 0.1, a—e: unidentified, f: NBD-OH.

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Ltd. (Tokyo, Japan). Amydae Carapax consisted of three samples, one from India obtained from Takasago Yakugyo Co., Ltd. and others were from unknown location and obtained from Tochimoto Tenkaido Co., Ltd. and Kinokuniya Kanyakkyoku Co., Ltd. Three samples of Asini Gelatinum, Cervi Parvum Cornu, Hippocampus, Kokurozin and Koukuzin were from China and were provided by Tenjin Li Sheng Pharmaceutical Factory (Tenjin, China).

Reference Standard L-Alanine (Ala), L-arginine monohydrochloride (Arg), L-aspartic acid (Asp), L-cystine (Cys–Cys), L-glutamic acid (Glu), glycine (Gly), L-hystidine monohydrochloride monohydrate (His), L-isoleucine (Ile), L-leucine (Leu), L-lysine monohydrochloride (Lys), L-methionine (Met), L-phenylalanine (Phe), L-proline (Pro), L-serine (Ser),

TABLE I. Free Amino Acid Contents[a]

Crude drugs[b]	α-AAA	α-ABA	Ala	Ala–Hcy	β-Ala	Ans	Arg	Asn	Asp	Car
Bezoar Bovis	ND	ND	0.01	ND—0.09	ND	ND	ND—0.01	ND—0.01	ND	ND—0.02
Ostreae Testa	ND	ND	ND	ND	ND	ND	ND—0.01	ND	ND	ND
Asini Gelatinum	ND	0.08—0.18	0.71—0.72	ND	0.03—0.07	0.07—0.09	0.16—0.18	0.27—0.29	0.14—0.21	0.02—0.03
Lumbricus	ND—0.01	ND—0.04	0.12—3.38	ND—0.09	ND	ND	0.05—1.18	0.16—1.26	0.07—0.93	0.01—0.23
Cicadae Periostracum	ND	ND	0.01—0.02	ND—0.26	ND	ND	0.01	ND—0.01	ND	ND—0.02
Amydae Carapax	ND	0.03—0.08	0.06—0.16	ND	ND	ND	0.12—0.25	0.06—0.08	0.04—0.09	0.04—0.08
Cervi Parvum Cornu	ND—0.01	ND—0.36	0.21—1.23	ND—0.09	ND—0.02	ND—0.10	0.05—0.32	0.08—0.38	0.05—0.18	ND—0.02
Hippocampus	0.01—0.02	0.05—0.07	1.88—2.11	0.14—0.20	ND	0.03—0.94	0.19—0.33	0.49—0.63	0.22—0.32	ND
Kokurozin[c]	ND	0.10—0.17	0.70—0.89	ND	ND	ND	0.13—0.16	0.18—0.23	0.24—0.36	ND
Koukuzin[d]	ND	0.01—0.03	0.35—0.41	ND	ND	0.03—0.06	0.19—0.28	0.16—0.21	0.19	0.01—0.02
Detection limit	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.02	0.01

Crude drugs[b]	Cit	Cys–Cys	Gln	Glu	Gly	Hcy–Hcy	His	Hylys	Hypro	Ile
Bezoar Bovis	ND—0.01	ND	ND	0.01—0.03	0.03—0.19	ND	0.10—0.34	0.05—0.12	ND	0.01
Ostreae Testa	ND	ND	ND	ND—0.01	ND—0.03	ND	ND—0.08	ND	ND	ND—0.01
Asini Gelatinum	0.01	ND	ND	0.04—0.06	0.89—0.99	0.16—0.73	0.52—1.58	ND—3.00	0.43—2.93	0.01—0.09
Lumbricus	0.01—0.08	ND—3.88	0.03—0.14	0.12—1.34	0.03—0.80	ND—0.25	0.07—1.23	ND—0.01	ND	0.06—0.81
Cicadae Periostracum	ND	ND	ND	0.01—0.02	ND	ND	0.04—0.17	ND	ND	ND—0.06
Amydae Carapax	0.01—0.06	ND	ND	0.12—0.17	0.16—0.33	0.18—0.89	0.41—0.61	ND	0.01—0.10	ND—0.11
Cervi Parvum Cornu	0.02—0.24	ND	ND—0.02	0.02—1.04	0.19—1.36	ND—0.14	0.42—2.41	ND—0.11	ND—0.26	0.04—0.17
Hippocampus	0.08—0.13	ND	ND—0.01	0.37—0.47	0.37—0.63	ND—0.15	0.66—1.15	0.26—0.39	0.01—0.05	0.35—0.48
Kokurozin[c]	0.01—0.09	ND	ND	0.21—0.25	0.23—0.28	ND	0.43—0.64	0.02—0.26	0.01—0.07	0.30—0.41
Koukuzin[d]	0.01	ND	0.01—0.02	0.07—0.11	0.18—0.21	ND—0.24	0.29—0.46	ND—0.07	0.01—0.05	0.10—0.20
Detection limit	0.01	1.09	0.01	0.01	0.02	0.07	0.02	0.01	0.01	0.01

Crude drugs[b]	Leu	Lys	1Mehis	3Mehis	Met	Orn	PEA	Phe	P-Ser
Bezoar Bovis	0.01—0.02	0.04—0.13	ND	ND	ND	0.02—0.19	ND	0.05—0.07	ND
Ostreae Testa	ND—0.01	ND—0.04	ND	ND	ND	ND—0.16	ND	ND—0.05	ND
Asini Gelatinum	0.12—1.04	ND	0.01	0.53—0.67	ND—0.02	ND	ND	0.25—1.77	ND—0.01
Lumbricus	0.06—1.46	0.12—1.37	ND—0.04	ND—0.06	ND—0.04	ND—0.08	ND—0.02	0.05—0.84	0.01—0.02
Cicadae Periostracum	0.01	0.02—0.05	ND	ND	ND	ND—0.19	ND	0.01—0.08	ND
Amydae Carapax	0.06—0.09	ND—0.53	ND	0.03—0.08	ND	ND—0.14	0.01—0.04	ND—0.12	0.01—0.02
Cervi Parvum Cornu	0.09—0.33	0.16—0.59	ND	ND—0.05	ND—0.03	0.05—0.34	0.01—0.04	0.14—0.26	ND
Hippocampus	0.47—0.67	0.81—3.40	0.02—0.03	0.03—0.05	0.02—0.09	0.08—0.13	ND—0.04	0.33—0.41	0.01
Kokurozin[c]	0.34—0.53	0.25—0.57	ND	ND—0.12	0.04—0.11	0.06—0.31	0.01—0.02	0.25—0.38	ND—0.01
Koukuzin[d]	0.22—0.42	0.64—0.68	ND—0.02	ND—0.02	ND—0.01	0.10—0.16	0.01	0.23—0.37	ND
Detection limit	0.01	0.02	0.01	0.01	0.01	0.02	0.01	0.01	0.01

Crude drugs[b]	Pro	Sar	Ser	Tau	Thr	Trp	Tyr	Val	Total
Bezoar Bovis	ND	ND	ND	0.14—0.31	ND—0.01	ND—0.01	ND—0.01	0.01—0.02	0.70— 0.99
Ostreae Testa	ND—0.02	ND	ND	ND	ND	ND	ND	ND—0.04	0.07—0.44
Asini Gelatinum	0.10—0.40	0.04—0.24	0.04	0.01	0.04—0.06	0.01—0.20	0.07—0.09	0.14—0.30	8.57—14.29
Lumbricus	0.03—0.37	ND	0.01—0.33	ND—0.01	0.03—0.79	0.07—0.10	0.01—0.56	0.13—1.22	1.37—22.77
Cicadae Periostracum	ND	ND	ND	ND	ND—0.01	ND—0.01	0.01—0.02	0.01—0.22	0.20— 1.06
Amydae Carapax	0.05—0.13	ND	0.01	0.02—0.10	0.02—0.04	0.01—0.19	0.02—0.03	0.05—0.07	2.69— 3.37
Cervi Parvum Cornu	0.09—1.05	ND	0.01	0.18—0.30	0.06—0.20	0.02—0.38	0.05—0.14	0.13—0.40	2.54—11.73
Hippocampus	0.38—4.30	0.05—0.11	0.01	0.76—1.01	0.32—0.67	ND—0.03	0.17—0.27	0.57—0.73	10.05—14.63
Kokurozin[c]	0.29—0.44	0.03—0.06	0.02—0.03	0.19—0.25	0.12—0.15	ND—0.01	0.19—0.24	0.15—0.17	4.87— 6.67
Koukuzin[d]	0.01—0.18	ND—0.02	0.04—0.05	0.02—0.03	0.09—0.11	ND—0.06	0.13—0.14	0.15—0.16	0.15— 0.16
Detection limit	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	

Unit: mg/kg crude drug. *a*) Amino acid contents after 1 h sonication in 0.1 N HCl, The Data are shown as "min.—max." values. *b*) From Namba's writing (Colored Illustration of Wakan-yaku), except for Kokurozin and Koukuzin. *c*) The external genitalia of male *Equus asinus* L. *d*) The external genitalia of male *Canis familiaris* L. ND: not detected.

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L-valine (Val) of special grade and L-carnosine (Car) of biochemical grade were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-2-Aminoadipic acid (α-AAA), L-2-amino-*n*-butyric acid (α-ABA), β-alanine (β-Ala), L-asparagine (Asn), L-citruline (Cit), L-glutamine (Gln), DL-homocystine (Hcy–Hcy), L-hydroxyproline (Hypro), L-ornithine monohydrochloride (Orn), *o*-phosphorylethanolamine (PEA), sarcosine (Sar) of special grade and taurine (Tau) of the first grade were obtained from Katayama Chemical Industries (Osaka, Japan). L(+)Cystathionine (Ala–Hcy), *o*-phospho-L-serine (P-Ser) of special grade were from Sigma (St. Louis, U.S.A.), and L-anserine nitrate (Ans), DL-plus *allo*-δ-hydroxylysine (Hylys), L-1-methylhistidine (1-Mehis), L-3-methylhistidine (3-Mehis) were from Calbiochem (San Diego, U.S.A.). Amino acid standards were dissolved in 0.1 N HCl for use. The final amino acid concentrations of the standard solution are given in Fig. 1.

Reagents The water which was used in this investigation was prepared using a Milli-RO 60 system using reverse osmosis and an ionization cartridge (Nihon Millipore, Tokyo, Japan). The chemicals used were analytical grade and the solvents chromatographic grade. Sodium dihydrogenphosphate·$2H_2O$, disodium hydrogen phosphate $12H_2O$, phosphoric acid, boric acid, sodium hydroxide, hydrochloric acid, *o*-phthalaldehyde (OPA),[3] 3-mercaptopropionic acid (3-MPA),[4] trichloroacetic acid (TCAA), acetonitrile (MeCN) and methanol (MeOH) were obtained from Katayama Chemical Industries; 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)[3] was from Nakarai Tesque (Kyoto, Japan).

Apparatus The HPLC system (Shimadzu, Kyoto, Japan) consisted of two LC-6AD pumps, a SCL-6B system controller for gradient and derivatization programming, a SIL-6B auto-injector and a CTO-6A column heater. HPLC system was used with an automated derivatization procedure to ensure proper reproducibility and quantification because OPA-amino acid adducts are unstable. Fluorescence was monitored with a FS-8010 fluorescence detector (Tosho, Tokyo, Japan). Data from the HPLC system was recorded continuously by a C-R5A data processor (Shimadzu). A YMC-Pack ODS-AM-302, 5 μm (150 × 4.6 mm) column and a YMC-Guardpack ODS-AM-302, 5 μm (10 × 4.0 mm) guard column were obtained from YMC Co., Ltd. (Kyoto, Japan).

Preparation of Sample Solutions for FAA A powdered sample (0.2 g) was mixed with 5 ml 0.1 N HCl and sonicated for 1 h, then 5 ml 10% TCAA was added and the mixture shaken well for 10 min.[5] After centrifugation at 4000 rpm for 5 min, the protein-free upper layer was used as the sample solution for FAA assay.

Preparation of Sample Solutions for TAA A powdered sample (0.2 g) was mixed with 5 ml 0.1 N HCl and sonicated for 1 h, then 5 ml water was added and the mixture shaken well for 10 min. After centrifugation at 4000 rpm for 5 min, 0.5 ml of the upper layer and 0.5 ml conc. HCl were transferred to a vial, and the vial was sealed after removal of the air under reduced pressure. The vial was kept in a dry oven (110 °C ± 5 °C) for 24 h to hydrolyze the proteins. After cooling, 0.1 ml of the hydrolyzate was mixed with 0.9 ml 0.1 M borate buffer (pH 9.0, adjusted with 4 M sodium hydroxide). The mixture was used as the sample solution for TAA assay.

Preparation of the Derivatization Agent[2,4] 20 μl 3-MPA was mixed with 15 ml 0.1 M borate buffer (pH 9.0). This mixture was reagent A. 20 mg OPA was dissolved in 5 ml MeOH, followed by the addition of 10 ml 0.1 M borate buffer (pH 9.0). This solution was reagent B. 0.1 g NBD-Cl was dissolved in 15 ml MeOH to give reagent C.

Analysis of Primary Amino Acids To 20 μl of the standard and sample solutions, 320 μl of reagent A, 320 μl of reagent B and 340 μl of water were added and mixed. The mixture was kept at 25 °C for 2 min, then 10 μl of the mixture was injected immediately into the column. The detector was set at an excitation wavelength of 345 nm and an emission wavelength of 450 nm. The column temperature was 45 °C and the flow rate was 1.0 ml/min. Solvent A consisted of 10 mM sodium phosphate buffer (pH 6.8) and MeCN (197 : 3). Solvent B consisted of 10 mM sodium phosphate buffer (pH 6.8) and MeCN (4 : 6). The gradient elution program was as follows; concentration of solvent B (time) from 2% (0 min), 20% (30 min) then 60% (60 min).

Analysis of Secondary Amino Acids To 40 μl of the standard and sample solutions, 320 μl of reagent A, 320 μl of reagent B and 320 μl of reagent C were added and mixed. The mixture was kept at 25 °C for 10 min, then 20 μl of the mixture was injected immediately into the column. The detector was set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. The column temperature was 40 °C and the flow rate was 1.0 ml/min. Solvent A consisted of 10 mM sodium phosphate buffer (pH 6.8) and MeCN (19 : 1). Solvent B consisted of 10 mM sodium phosphate buffer (pH 6.8) and MeCN (2 : 8). The gradient elution program was as follows: concentration of solvent B (time) from 10% (0 min) to 100% (4 min).

Results and Discussion

Typical chromatograms of primary and secondary amino acids obtained from a standard solution is shown in Fig. 1. It shows the good separation of the amino acids from each other and from materials originating from the reagents.

Calibration curves of all amino acids were linear over the concentration range 0—0.5 μmol/ml. The reproducibility was good with a C.V. between 2 to 5% except for His (7%) which exhibited an inferior peak shape compar-

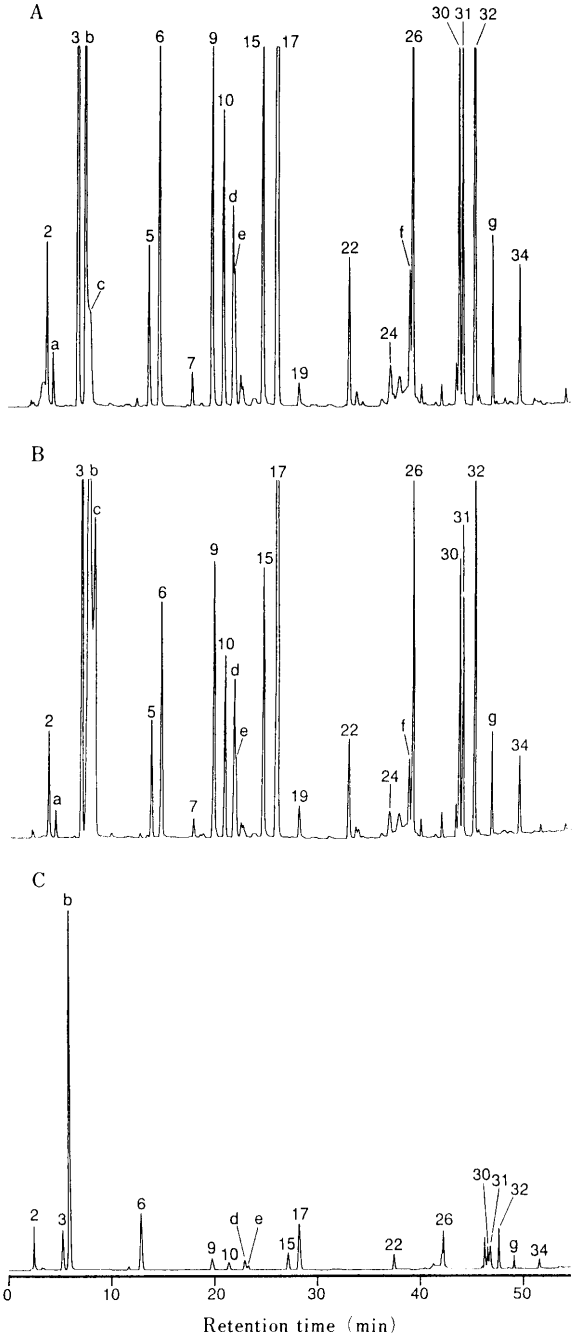

Fig. 2. Chromatograms of Primary Free Amino Acids Obtained from Sample Solutions of Lumbricus

A: sample 1. B: sample 2. C: sample 3. 2—34: see Fig. 1. a—g: unidentified.

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ed with the others. Table I shows the FAA content of 10 different animal crude drugs for 3 samples giving minimum and maximum values. The results showed that the FAA content was less than 3% in these samples and that Lumbricus, Asini Gelatinum and Hippocampus contained more FAA than the others while Ostreae Testa, Bezoar Bovis and Cicadae Periostracum contained less FAA.

Bezoar Bovis contained much more taurine, 27% of the total FAA contents, which was remarkable compared with the figure for other crude drugs of less than 7%. That was because of the taurine and taurocholic acid in the bile of cattle, and which is considered to be the source of Bezoar Bovis's origin.

The FAA content of Lumbricus and Cervi Parvum Cornu varied depending on the sample, although they were all from the same habitat. Chromatograms of primary FAA obtained from a Lumbricus sample solution is shown in Fig. 2.

Jung *et al.*[6] reported that Cervi Parvum Cornu exhibited some variations in the FAA composition depending on the habitat, species of original animal and the tissue sampled. However, this was not true for the

TABLE II. Total Amino Acid Contents[a]

Crude drugs[b]	α-AAA	α-ABA	Ala	Ala-Hcy	β-Ala	Ans	Arg	Asn	Asp	Car
Bezoar Bovis	ND	ND	0.07— 0.11	ND	ND	ND	0.11— 0.13	0.05— 0.09	0.05— 0.11	ND
Ostreae Testa	ND	ND	ND— 0.06	ND	ND	ND	ND	ND	ND— 0.06	ND
Asini Gelatinum	ND—0.06	4.49—5.33	60.19—68.59	ND	ND	1.10—1.33	61.21—71.67	34.01—38.88	34.18—39.28	0.05—0.07
Lumbricus	ND	ND—0.09	8.60—12.88	ND	ND	ND	13.52—16.49	10.50—13.96	13.78—14.98	ND
Cicadae Periostracum	ND	ND	0.70— 0.89	ND	ND	ND	0.41— 0.56	0.60— 0.92	0.90— 1.19	ND
Amydae Carapax	ND	ND—0.07	4.50— 5.65	ND	ND	ND	4.90— 6.30	3.10— 4.21	2.73— 3.35	ND
Cervi Parvum Cornu	ND	ND—0.73	7.03— 9.57	ND	ND	ND	7.41— 9.58	3.38— 4.38	4.19— 5.45	ND
Hippocampus	ND	0.09—0.14	8.96—15.33	0.63—2.05	ND	1.70—2.45	0.95—12.93	3.79— 5.76	4.58— 7.34	ND
Kokurozin[c]	ND	0.35—0.41	13.70—14.69	ND—0.67	ND	ND	14.32—15.11	7.44— 7.55	8.40— 8.82	ND
Koukuzin[d]	ND	ND	2.48— 3.12	ND	ND	ND	2.62— 3.27	1.26— 1.56	1.60— 2.03	ND
Detection limit	0.05	0.03	0.03	0.09	0.03	0.08	0.06	0.05	0.04	0.04

Crude drugs[b]	Cit	Cys-Cys	Gln	Glu	Gly	Hcy-Hcy	His	Hylys	Hypro	Ile
Bezoar Bovis	ND	ND	ND	0.15— 0.21	0.25— 1.52	ND	0.63— 4.16	0.26—0.87	ND	ND
Ostreae Testa	ND	ND	ND	ND— 0.09	ND— 0.14	ND	ND	ND	ND	ND
Asini Gelatinum	0.08—0.10	14.38— 26.99	ND	61.49—72.95	97.25—107.94	ND	218.92—233.37	0.13—0.21	64.06—75.08	10.15—11.64
Lumbricus	ND	15.34— 27.56	ND	19.66—25.45	8.07— 9.05	ND	13.56— 14.48	ND—0.19	0.57— 1.00	6.80— 7.89
Cicadae Periostracum	ND	ND	ND	1.13— 1.60	0.60— 0.92	ND	ND— 1.46	ND	ND	0.45— 0.89
Amydae Carapax	ND	51.34— 70.46	ND	5.14— 6.60	11.14— 13.99	ND	15.56— 19.12	ND	4.76— 8.65	0.93— 1.32
Cervi Parvum Cornu	ND	36.68—113.59	ND	7.88— 9.64	16.04— 22.13	ND	22.02— 32.47	0.07—0.27	5.28—10.63	1.42— 1.86
Hippocampus	0.09—0.11	ND— 17.49	ND	7.47—12.41	15.75— 27.00	ND	20.56— 34.18	0.17—0.27	6.30— 8.86	1.73— 2.95
Kokurozin[c]	0.08—0.10	87.60—100.56	ND	16.45—16.67	31.17— 34.83	2.23—2.98	41.51— 43.84	0.15—0.23	13.73—16.97	2.91— 3.04
Koukuzin[d]	ND	22.91— 41.62	ND	2.85— 3.73	6.11— 7.62	ND	8.72— 9.53	ND	12.23—16.93	0.52— 0.67
Detection limit	0.05	10.93	0.09	0.04	0.02	0.82	0.21	0.10	0.08	0.03

Crude drugs[b]	Leu	Lys	1Mehis	3Mehis	Met	Orn	PEA	Phe	P-Ser
Bezoar Bovis	0.11— 0.19	0.51— 0.78	ND	ND	ND	1.29—1.39	ND	0.51— 0.85	ND
Ostreae Testa	ND— 0.07	0.34— 0.58	ND	ND	ND	ND	ND	0.64— 0.77	ND
Asini Gelatinum	16.92—19.42	31.98—33.16	0.24—0.30	0.55—0.74	5.01—5.91	2.70—3.41	ND	14.36—16.54	ND
Lumbricus	8.75—10.84	16.43—16.58	0.14—0.17	ND—0.14	1.70—2.76	1.84—6.07	ND	6.60— 6.42	ND
Cicadae Periostracum	0.34— 0.49	ND— 1.21	ND—0.35	ND	ND	ND—0.95	ND	0.63— 1.15	ND
Amydae Carapax	1.20— 1.49	3.04— 3.50	ND	ND	ND—0.12	ND	ND	1.56— 1.68	ND
Cervi Parvum Cornu	2.16— 2.73	4.56— 6.14	ND	ND	0.05—0.37	0.63—1.21	ND	2.00— 2.69	ND
Hippocampus	2.03— 3.31	5.08— 7.50	0.07—0.08	ND	1.55—2.93	1.17—1.68	0.21—0.25	2.21— 3.32	ND
Kokurozin[c]	4.35— 4.74	8.35—10.87	ND	0.32—0.41	0.56—0.63	1.21—1.66	ND	3.74— 4.27	ND
Koukuzin[d]	0.77— 0.94	1.77— 1.85	ND	ND	0.12—0.16	ND	ND	0.72— 0.88	ND
Detection limit	0.03	0.22	0.05	0.08	0.04	0.31	0.08	0.04	0.07

Crude drugs[b]	Pro	Sar	Ser	Tau	Thr	Trp	Tyr	Val	Total
Bezoar Bovis	ND	ND	ND	0.45—1.46	ND— 0.04	ND	ND—0.07	0.06— 0.13	5.09— 10.18
Ostreae Testa	ND	ND	ND	ND	ND	ND	ND	ND	1.16— 1.44
Asini Gelatinum	72.51—80.72	12.08—12.73	ND	ND—0.03	11.60—13.11	1.95—2.29	4.97—5.75	18.50—20.72	862.10—967.25
Lumbricus	2.08— 4.61	ND	ND	ND	6.49— 7.21	ND	4.93—4.80	7.24— 8.45	182.04—208.20
Cicadae Periostracum	1.01— 1.46	ND	ND	ND	0.43— 0.58	ND	0.40—0.95	0.61— 0.85	9.80— 14.86
Amydae Carapax	5.83— 9.49	ND	ND	ND	1.19— 1.43	ND—0.18	0.46—0.54	1.13— 1.42	129.48—157.64
Cervi Parvum Cornu	6.19—11.91	ND— 1.41	ND	0.18—0.26	1.59— 2.12	0.25—0.43	0.64—0.79	2.06— 2.83	148.67—251.91
Hippocampus	8.93—12.22	ND	ND	0.60—0.95	2.12— 3.36	ND	0.95—1.39	3.23— 5.37	122.60—190.24
Kokurozin[c]	16.40—20.67	1.80— 2.66	ND	0.24—0.28	2.97— 3.28	0.37—0.63	1.31—1.44	4.68— 5.24	670.25—707.29
Koukuzin[d]	13.56—19.36	1.78— 2.73	ND	ND	0.62— 0.78	ND	0.27—0.37	0.76— 1.02	93.26—106.68
Detection limit	0.15	0.30	0.03	0.03	0.04	0.06	0.05	0.03	

Unit: mg/g crude drug. *a*) Amino acid contents after 1 h sonication in 0.1 N HCl and 24 h hydrolysis in 6 N HCl at 110±5 °C, The data are shown as "min.—max." values. *b—d*) See Table I. ND: not detected.

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FAA content of Lumbricus. In this determination, the sample solution was prepared from Lumbricus samples from the same habitat. Accordingly, it was considered that the very wide range of FAA contents was due to differences in the method of pretreatment and/or preservation.

Table II shows the TAA content of the 10 kinds of animal crude drugs in 3 samples with minimum and maximum values. The results showed that Asini Gelatinum (about 90% TAA) and Kokurozin (about 70%) contained a substantial amount of TAA while Bezoar Bovis, Ostreae Testa and Cicadae Pestracum had a TAA content of less than 2%. Compared with the FAA contents, the TAA content of every sample was found to lie within a more narrow range, especially the TAA content of Anisi Gelatinum, Lumbricus, Kokurozin and Koukuzin which exhibited very little variation.

Bezoar Bovis contained much more taurine about 13%

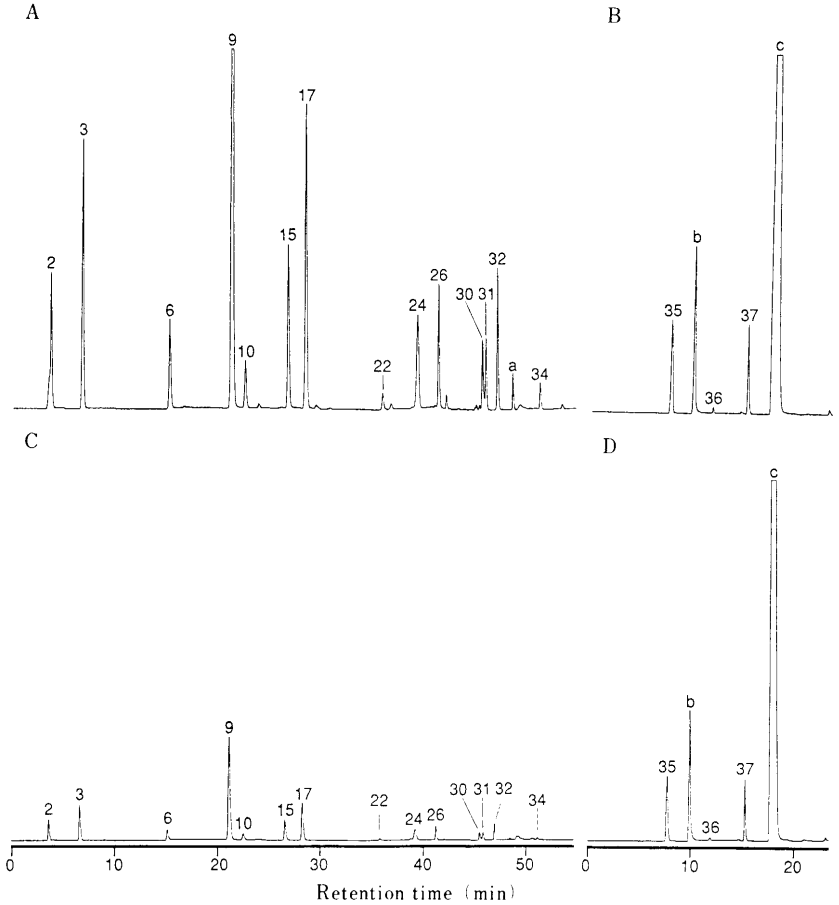

Fig. 3. Typical Cheomatograms of Amino Acids Obtained from Sample Solution of Kokurozin and Koukuzin

A: primary amino acids of kokurozin. B: secondary amino acids of kokurozin. C: primary amino acids of koukuzin. D: secondary amino acids of koukuzin., 2—37: see Fig. 1. a, b: unidentified, c: NBD-OH. Kokurozin and Koukuzin: see Table I.

Table III. Total Amino Acid Molar Ratio[a)]

Crude drugs[b)]	α-AAA	α-ABA	Ala	Ali-Hcy	β-Ala	Ans	Arg	Asn	Asp	Car	Cit	Cys-Cys	Gln	Glu	Gly	HcY-Hcy	His	Hylys
Asini Gelatinum	—	—	53	—	—	—	23	18	20	—	—	6	—	34	100	—	78	—
Lumbricus	—	1	111	—	—	—	64	74	95	—	—	83	—	136	100	—	174	1
Amydae Carapax	—	1	34	—	—	—	16	14	14	—	—	157	—	24	100	—	51	—
Cervi Parvum Cornu	—	3	24	—	—	—	15	10	14	—	—	143	—	24	100	—	50	—
Hippocampus	—	—	49	2	—	3	17	11	16	—	1	25	—	24	100	—	49	1
Kokurozin[c)]	—	1	36	—	—	—	16	11	15	—	1	88	—	25	100	2	46	—
Koukuzin[d)]	—	—	34	—	—	—	15	10	15	—	—	150	—	25	100	—	48	—

Crude drugs[b)]	Hypro	Ile	Leu	Lys	1Mehis	3Mehis	Met	Orn	PEA	Phe	P-Ser	Pro	Sar	Ser	Tau	Thr	Trp	Tyr	Val
Asini Gelatinum	38	6	10	12	—	—	3	1	—	7	—	49	10	—	8	8	1	2	12
Lumbricus	5	49	67	79	1	1	14	19	—	35	—	29	—	—	—	51	—	23	60
Amydae Carapax	33	5	6	11	—	—	—	—	—	6	—	43	—	—	—	7	—	2	7
Cervi Parvum Cornu	24	5	7	12	—	—	1	2	—	6	—	32	5	—	1	6	1	2	8
Hippocampus	13	6	7	13	—	—	5	3	1	6	—	33	—	—	—	8	—	2	13
Kokurozin[c)]	27	5	8	12	—	—	1	2	—	5	—	37	6	—	—	6	1	2	9
Koukuzin[d)]	128	5	8	12	—	—	1	—	—	5	—	163	29	—	—	6	—	2	8

a) The data are given as the mean of 3 samples and the relative percentage with Gly as 100%. *b*—*d*) See Table I. —: Less than 1.

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of the total TAA content, than other crude drugs which had less than 1%. As in the case of FAA, this was related to its origin. As would be expected, Ostereae Testa originating from a shell of *Ostrea gigas* THUNBERG (Ostreidae) contained very little FAA and TAA. Asini Gelatinum contained about 1% FAA and about 90% TAA, the FAA and TAA contents were influenced by the origin of the materials and their pretreatments.

It was difficult to distinguish Kokurozin from Koukuzin because both came from the same part of different species of animal. In this determination, there were practically no differences in the FAA content, however, the TAA content showed large differences; about 70% Kokurozin and about 10% Koukuzin. Furthermore, the ratio of secondary amino acids to TAA was marked; about 5% for Kokurozin and about 33% for Koukuzin. These results indicate that TAA determination makes it possible to distinguish Kokurozin from Koukuzin. Typical chromatograms of the amino acids obtained from Kokurozin and Koukuzin sample solutions are shown in Fig. 3.

The TAA molar ratios are shown in Table III except for Bezoar Bovis, Ostreae Testa and Cicadae Periostracum which have very low TAA contents. It was found that Lumbricus had a characteristic ratio as shown in Table III.

From the results, it was shown that FAA and TAA were characteristic for the animal crude drugs studied. Therefore, determination of FAA and TAA under the established standard condition would be a promising method for evaluating animal crude drugs.

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