Studies on the "Signal" Constituents for the Evaluation of Animal Crude Drugs. III.[1] Nucleic Acid Components

Akio HASHIMOTO,* Takashi NAKAMURA, Yoshio KOKUSENYA, Hideo NAKAI, and Tadashi SATO

Analytical Research Laboratory, Tanabe Seiyaku Co., Ltd., 16–89 Kashima 3-chome, Yodogawa-ku, Osaka 532, Japan. Received July 4, 1996; accepted November 21, 1996

An analytical method for determining the contents of 7 nucleic acid bases, 6 ribonucleosides and 5 deoxyribonucleosides in animal crude drugs were established. The contents in free nucleic acid components (the ice-cold extracts) and in total nucleic acid components (the heated extracts) of 8 crude drugs were quite different from one another, indicating that these content pattrens could be used as a "signal" of each animal crude drug. For example, the contents of inosine in Lumbricus, uracil in Cervi Parvum Cornu, and guanine and hypoxanthine in Hippocampus were high. The total contents in each crude drug were about twice or three times as large as the free contents. The main degradation products by the heated extracts were adenine and guanine.

These results suggested that the contents of nucleic acid components could be one of the "signal" constituents for evaluation of animal crude drugs.

Key words animal crude drug; nucleic acid base; nucleoside; HPLC

Animal crude drugs have been widely used in various Chinese medicines. However, there are few studies evaluating the constituents of these drugs as compared to studies on plant crude drugs. It is very important to quantify and/or qualify the contents of the constituents of animal crude drugs from the viewpoint of quality control. We have been investigating the "signal" constituents to help in such evaluation and reported earlier that amino acids and organic acids were useful "signal" constituents to evaluate animal crude drugs.[2,3]

It was reported that uracil, uridine, and hypoxanthine were contained in Cervi Parvum Cornu.[4] Hypoxanthine is thought to be one of the medicinally active components in this drug.[5] All three substances are nucleic acid components, and it is assumed that their contents may differ in various animal crude drugs.[6] We presumed that the nucleic acid components might be "signal" constituents, like amino acids and organic acids. In this paper, the analytical method for determining the contents of 7 nucleic acid bases, 6 monoribonucleosides and 5 deoxyribonucleosides in animal crude drugs was established, and the contents in ice-cold extracts (of free nucleic acid components) and heated extracts (of total nucleic acid components) of 8 crude drugs were determined using this method.

Experimental

Samples The samples used were as follows: three board samples (about 9 × 4 × 1 cm) of Asini Gelatinum from China, which met the requirements of the Japanese Standard of Pharmaceutical Ingredients 1989 (JSPI)[7] and which showed the label "凶东阿胶" on each surface [one was obtained through Nihon Funmatsu Yakuhin Co., Ltd. (Osaka, Japan), and the others were provided by Tenjin Li Sheng Pharmaceutical Factory (Tenjin, China)]; nine samples of Lumbricus: Lumbricus kwangtungensis, which met the requirements of JSPI (six were from Thailand and the others were from China, all samples were obtained through Nihon Funmatsu Yakuhin Co., Ltd.); three samples of Cicadae Periostracum from China, which met the requirements of JSPI [samples were obtained through Kinokuniya Kanyakkyoku Co., Ltd. (Tokyo, Japan), Takasago Yakugyo Co., Ltd. (Osaka) and Tochimoto Tenkaido Co., Ltd. (Osaka)]; three samples of Amydae Carapax, which met the requirements of JSPI (one was from India and obtained through Takasago Yakugyo Co., Ltd., and the others were from an unknown habitat and obtained through Tochimoto Tenkaido Co., Ltd. and Kinokuniya Kanyakkyoku Co., Ltd.); six samples of Cervi Parvum Cornu: *Cervus elaphus* L. var. *xanthopygus* MILNE-EDWARDS (all the samples were from China and obtained through Nihon Funmatsu Yakuhin Co., Ltd.); and three samples of Hippocampus: *Hippocampus trimaculatus* LEACH; three samples of Kokurozin: the external genitalia of male *Equus asinus* L.; and three samples of Koukuzin: the external genitalia of male *Canis familiaris* L. (all the samples were from China and provided by Tenjin Li Sheng Pharmaceutical Factory). These samples were identified and tested by each maker, and stored in a tightly stoppered glass bottle at 25 °C until the quantitative analysis.

Reference Standard Adenine hydrochloride (Ade), adenosine (Ado), cytosine (Cyt), cytidine (Ctd), 2′-deoxyadenosine (dAdo), 2′-deoxycytidine hydrochloride (dCtd), guanine (Gua), hypoxanthine (Hyp), inosine (Ino), xanthine (Xan), thymine (Thy), thymidine (Thd), uracil (Ura), and uridine (Urd), which were of more than 99% purity, were obtained from Kojin Co., Ltd. (Kyoto, Japan). 2′-Deoxyguanosine (dGuo) and xanthosine (Xao), which were obtained from Nacalai Tesque Co., Ltd. (Kyoto), and 2′-deoxyinosine (dIno), which was obtained from Sigma Chemical Company (St. Louis, U.S.A.), were also of more than 99% purity. Sodium inosinate (IMP), which was the first grade, was obtained from Katayama Chemical Industries Co., Ltd. (Osaka).

Reagents Perchloric acid (60%), potassium carbonate, sodium dihydrogen phosphate·$2H_2O$ and phosphoric acid, which were of analytical reagent grade, and MeCN and MeOH, which were of HPLC grade, were purchased from Katayama Chemical Industries Co., Ltd..

Apparatus The HPLC system used (Shimadzu Co., Ltd., Kyoto) consisted of two LC-6AD pumps, a SCL-6B system controller, a SIL-6B auto-injector, a CTO-6A column heater, a SPD-6A UV detector, a C-R5A data processor, and a SPD-M6A photodiode array UV-VIS detector. YMC-Guardpack ODS-AM-302 (5 μm, 4.6 i.d. × 10 mm) and YMC-Pack ODS-AM-302 (5 μm, 4.6 i.d. × 150 mm) were obtained from YMC Co., Ltd. (Kyoto). Ekicrodisc 13 disposable filters (pore size 0.45 μm) were obtained from German Science Japan Co., Ltd. (Tokyo).

Preparation of Sample Solutions for the Free Nucleic Acid Components[8] First, each crude drug was powdered. Then, each powdered sample (0.2 g) was weighed into a test tube and mixed with 2 ml of ice-cold water and sonicated for 10 min in an ice-bath. Two ml of ice-cold 8% aqueous perchloric acid was added to the test tube, and the test tube was cooled with ice for 10 min. After cooling, the mixture was centrifuged at 3500 r.p.m. for 10 min at 0 °C. Two ml of the supernatant liquid was mixed with 0.3 ml of 2 M aqueous potassium carbonate, and the mixture was cooled with ice for 10 min to form a precipitate of potassium perchlorate. After cooling, the mixture was filtered, and the filtrate was used as the sample solution for the free nucleic acid components assay.

Preparation of Sample Solutions for the Total Nucleic Acid Com-

* To whom correspondence should be addressed.

ponents[8] Each crude drug was powdered, and each powdered sample (0.2 g) was weighed into a test tube, mixed with 2 ml of water and sonicated for 10 min. Two ml of 8% aqueous perchloric acid was added to the test tube, and the test tube was heated at 90 °C for 15 min to extract the total components as a free form. After ice cooling, the mixture was centrifuged at 3500 r.p.m. for 10 min. A two ml aliquot of the supernatant liquid was then mixed with 0.3 ml of 2 M aqueous potassium carbonate, the mixture was cooled with ice for 10 min, then filtered and the filtrate was used as the sample solution for the total nucleic acid components assay.

Preparation of Standard Solution Reference standards (0.1 mmol) were dissolved in 4% aqueous perchloric acid (100 ml), a 2 ml aliquot of this solution was then mixed with 0.3 ml of 2 M aqueous potassium carbonate, the mixture was cooled with ice for 10 min, then filtered and the filtrate was used as the standard solution for assay.

HPLC Analysis Components were identified by comparing the UV spectrum and retention time of each peak obtained in the sample solution with those in standard solution, because of the characteristic pattern of the UV spectrum of each component. Each content was calculated as the content in a dried crude drug, which was transfered from the sampling weight using the resulting value of "Loss on Drying" of "Crude Drugs" under the general test in "The Pharmacopoeia of Japan, 12th edition."[9]

Operating Conditions: injection volume; 10 μl: wavelength; 254 nm: column temperature; 30 °C: flow rate; 0.7 ml/min: two mobile phase, solvent A; 0.1 M sodium phosphate buffer (pH 3.8), solvent B; solvent A, MeCN and MeOH (8 : 1 : 1).

Gradient Program: a linear gradient from 3% B to 65% B in 25 min; a linear gradient from 65% B to 100% B in 5 min; 10 min at 100% B; from 100% B to 3% B in 5 min; 10 min at 3% B.

Results and Discussion

In the preliminary investigation nucleic acid bases, mononucleosides, and monodeoxyribonucleosides were detected in animal crude drugs, while there were few monoribonucleotides and monodeoxyribonucleotides. Therefore, we focus here on the determination of the contents of nucleic acid bases, ribonucleosides and deoxyribonucleosides.

The chromatogram of the standard solution containing nucleic acid components is shown in Fig. 1. Most of them were well separated within 23 min. Calibration curves over the concentration range 0—0.5 μmol/ml showed good linearity, and the reproducibility with 5 repetitions was good with relative standard deviation values of 0.88—1.06%.

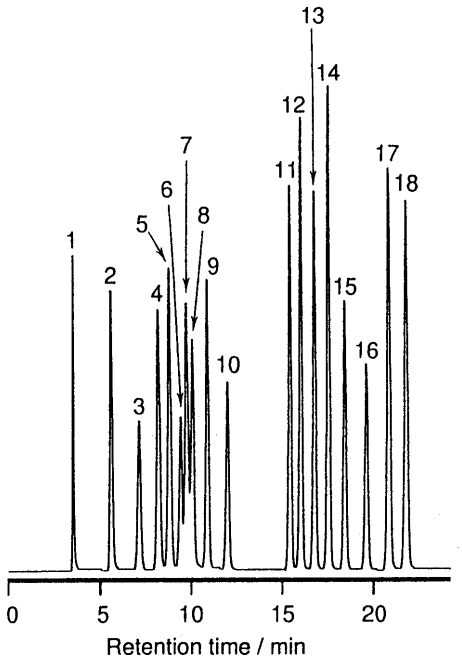

Fig. 1. Chromatogram of a Standard Mixture

1: Cyt, 2: Ura, 3: Ctd, 4: Gua, 5: Hyp, 6: dCtd, 7: Ade, 8: Xan, 9: Urd, 10: Thy, 11: Ino, 12: Guo, 13: dIno, 14: dGuo, 15: Xao, 16: dThd, 17: Ado, 18: dAdo.

The extraction for the determination of total nucleic acid components was carried out under heating at 90 °C. The stability of these components during heating was carefully checked because decomposition during heating is possible. The seven nucleic acid bases, cytidine, uridine, and thymidine were little decomposed, and there was no problem in the quantitative determination. However, deoxycytidine, and 7 purine nucleosides were hydrolyzed, and changed quantitatively into corresponding nucleic acid bases. All nucleosides except for cytidine, uridine, and thymidine, could be evaluated as corresponding nucleic acid bases.

The Free Nucleic Acid Components Contents It is thought that the free nucleic acid components in animal crude drugs increase with the degradation during pretreatment and preservation, compared with these components in living animals. These values may reflect the origins of animal crude drugs, the pretreatment process and preservation conditions. They are summarized in Table 1. More than ten components were detected from Asini Gelatinum, Lumbricus, Cervi Parvum Cornu, Hippocampus, Kokurozin and Koukuzin. The main components of each animal crude drug are as follows: inosine and hypoxanthine in Lumbricus, uracil in Cervi Parvum Cornu, guanine and hypoxanthine in Hippocampus. The components in Koukuzin were the same as these in Kokurozin except for deoxyinoshine. However, the contents of adenine, adenosine, guanine, guanosine, hypoxanthine, thymine, uracil, and xanthine in Kokurozin were clearly different from that in Koukuzin.

Few contents were detected from Asini Gelatinum, Cicadae Periostracum and Amydae Carapax, which makes it difficult to evaluate these crude drugs by their free nucleic acid components.

From the results in Table 1, it was concluded that each animal crude drug could be distinguished by comparing the content pattern of its free nucleic acid components.

The Total Content of Nucleic Acid Components The total content of nucleic acid components can be calculated as the sum of the free nucleic acid components in living animals and the products degraded by the heating extraction. It is suggested that these contents may also show characteristic patterns, and may be useful for the evaluation of each animal crude drug (Table 2). The content of the total nucleic acid components is twice that of the free contents in Lumbricus. The main components of the former were adenine, guanine and hypoxanthine. Characteristic of Lumbricus was the high content of hypoxanthine, which can be ascribed to the hydrolysis of inosine. The total content of nucleic acid components of Cervi Parvum Cornu was one and half times the free contents; the main components were guanine, uracil and hypoxanthine. Characteristic of Cervi Parvum Cornu was the high content of uracil and guanine. The total content of nucleic acid components of Hippocampus was twice that of the free contents, and the main components were guanine and hypoxanthine; characteristic of Hippocampus

Table 1. Content of Free Nucleic Acid Components in Animal Crude Drugs[a]

Crude drug[b]	n[c]	Ade	Ado	dAdo	Cyt	Ctd	dCtd	Gua	Guo	dGuo	Hyp
Asini Gelatinum	3	0.88±0.61	ND[2]	ND	ND	0.05±0.08	ND	0.60±0.33	0.23±0.17	ND	0.14±0.06
Lumbricus	9	0.44±0.18	0.53±0.39	ND	0.67±0.17	0.52±0.18	ND	1.45±1.71	0.79±0.23	0.15±0.10	3.26±3.29
Cicadae Periostracum	3	ND	ND	ND	ND	ND	ND	0.09±0.09	ND	ND	0.02±0.04
Amydae Carapax	3	ND	ND	ND	0.03±0.06	ND	ND	0.21±0.07	ND	ND	0.26±0.19
Cervi Parvum Cornu	6	0.03±0.02	ND	ND	0.17±0.04	0.08±0.07	ND	1.49±0.38	1.30±0.66	0.12±0.07	2.01±0.29
Hippocampus	3	0.10±0.03	0.06±0.02	ND	1.46±0.15	ND	ND	5.70±1.26	0.05±0.02	0.04±0.01	4.78±0.25
Kokurozin	3	0.11±0.02	0.05±0.01	ND	0.30±0.06	ND	ND	0.11±0.03	0.10±0.03	0.04±0.01	0.95±0.15
Koukuzin	3	0.44±0.02	0.12±0.01	ND	0.29±0.02	ND	ND	0.24±0.02	0.27±0.02	0.07±0.01	0.36±0.03
Detection limit		0.04	0.03	0.03	0.09	0.08	0.08	0.04	0.03	0.03	0.04

Crude drug[b]	Ino	dIno	Thy	dThd	Ura	Urd	Xan	Xao	Total
Asini Gelatinum	0.04±0.03	ND	0.16±0.02	0.07±0.12	0.27±0.09	0.16±0.10	0.08±0.02	ND	2.68±1.23
Lumbricus	7.85±3.01	0.31±0.21	0.24±0.11	0.22±0.18	2.13±0.43	0.96±0.32	0.51±0.16	0.04±0.04	20.06±3.92
Cicadae Periostracum	ND	ND	ND	ND	ND	ND	ND	ND	0.11±0.10
Amydae Carapax	0.04±0.08	ND	0.04±0.04	ND	0.10±0.03	0.03±0.03	0.14±0.08	ND	0.86±0.38
Cervi Parvum Cornu	0.88±0.42	0.16±0.08	0.21±0.08	0.15±0.10	3.37±0.79	1.56±0.76	0.67±0.16	0.08±0.02	12.28±2.76
Hippocampus	0.32±0.10	0.04±0.04	0.28±0.08	ND	1.25±0.14	0.15±0.02	0.85±0.10	ND	15.07±1.79
Kokurozin	0.16±0.04	0.06±0.02	0.67±0.09	0.09±0.02	1.96±0.27	0.29±0.06	1.76±0.33	0.13±0.02	6.80±1.13
Koukuzin	0.10±0.01	ND	0.21±0.00	0.09±0.00	0.88±0.08	0.33±0.02	0.64±0.05	0.08±0.01	4.13±0.26
Detection limit	0.04	0.04	0.06	0.06	0.05	0.04	0.05	0.05	

a) Unit: μmol/g dried weight. Free components after extraction with ice-cold 4% $HClO_4$. The data are given as the mean±S.D. value of all samples. *b*) Latin names used are from Namba's writing "Colored Illustrations of Wakan-yaku," except for Kokurozin and Koukuzin. *c*) Number of samples. ND: not detected.

Table 2. Content of Total Nucleic Acid Components in Animal Crude Drugs[a]

Crude drug[b]	n[c]	Ade	Cyt	Ctd	Gua	Hyp	Thy
Asini Gelatinum	3	1.69±1.03	0.54±0.19	0.19±0.09	1.35±0.76	0.22±0.07	0.72±0.03
Lumbricus	9	6.17±2.47	1.15±0.25	1.99±1.28	6.53±1.55	14.40±2.22	0.34±0.19
Cicadae Periostracum	3	0.11±0.03	0.66±0.04	ND	0.27±0.09	0.08±0.05	ND
Amydae Carapax	3	0.45±0.24	0.13±0.03	ND	0.61±0.27	0.37±0.30	0.04±0.04
Cervi Parvum Cornu	6	2.62±0.52	0.26±0.05	0.02±0.04	5.64±1.19	3.62±0.82	0.27±0.09
Hippocampus	3	1.46±0.07	1.93±0.18	1.21±0.20	11.98±2.22	6.48±0.37	0.36±0.09
Kokurozin	3	4.80±0.33	0.92±0.03	1.01±0.11	4.95±0.32	1.99±0.13	1.11±0.09
Koukuzin	3	2.98±0.25	0.61±0.06	0.65±0.13	2.91±0.13	0.72±0.04	0.37±0.13
Detection limit		0.04	0.09	0.08	0.04	0.04	0.06

Crude drug[b]	dThd	Ura	Urd	Xan	Total
Asini Gelatinum	0.25±0.08	0.40±0.21	0.25±0.18	0.15±0.03	5.74±2.00
Lumbricus	0.01±0.03	1.48±1.03	1.47±0.52	2.51±0.69	36.06±3.82
Cicadae Periostracum	ND	0.02±0.03	ND	0.05±0.01	1.18±0.17
Amydae Carapax	ND	0.10±0.02	0.02±0.03	0.41±0.17	2.14±1.01
Cervi Parvum Cornu	0.18±0.19	3.99±1.13	1.80±0.92	1.08±0.30	19.50±4.34
Hippocampus	0.02±0.03	1.32±0.19	0.19±0.02	1.90±0.07	26.86±2.59
Kokurozin	0.17±0.02	2.41±0.16	0.61±0.17	4.54±0.25	22.49±1.29
Koukuzin	0.14±0.04	1.05±0.25	0.53±0.06	1.33±0.11	11.30±0.39
Detection limit	0.06	0.05	0.04	0.05	

a) Unit: μmol/g dried weight. Total components after 15 min hydrolysis in 4% $HClO_4$ at 90 °C. The data are given as mean±S.D. value of all samples. *b, c*) See Table 1. ND: not detected.

was the high content of guanine. The total contents in Kokurozin and in Koukuzin were three times as large as their free contents, respectively; the main components in both of them were adenine and guanine, and the total contents in Kokurozin were twice as large as those in Koukuzin.

It was difficult to evaluate the total contents of Asini Gelatinum, Cicadae Periostracum and Amydae Carapax, as true of the free nucleic acid components.

From the composition of the contents of free and total nucleic acid components, it was determined that the contents of adenine and guanine had increased. This indicated that these two nucleic acid bases exist as a complex, such as nucleic acids, oligonucleotides and so on.

The Difference of the Free Components in Lumbricus The free contents in Lumbricus were found to be different in the samples from China and those from Thailand (Table 3).

The contents of inosine and hypoxanthine also differed even from the same place of production, Thailand (Fig. 2). These differences in a single animal crude drug, *i.e.* Lumbricus, indicates that there must be differences in the origin, pretreatment process and preservation conditions. Since all samples used were Lumbricus kwangtungensis, the difference must reflect the pretreatment process or preservation conditions. The effect of storage conditions was investigated by storing powdered samples of Lumbricus under the following conditions: 25 °C, tightly closed containers; 40 °C, tightly closed containers; and 40 °C–75% relative humidity, open containers. Changes in the free and total contents were determined after 1 and 3 months storage to learn whether or not the free components were produced during the preservation. Little change was observed in the contents of any sample stored under any of the above conditions. This indicated that the differences in the free contents were due to the pretreatment process.

According to the "Coloured Illustrations of Wakan-Yaku" by Namba,[10] the pretreatment method is as follows: raw materials are dipped in a slightly frothy solution which is a mixture of straw ashes and hot water to remove the outer mucous membrane. Then, the abdomen is cut open and the contents removed. After washing with hot water, the materials are dried in sunlight or by the fire. If the pretreatment was done by the described method, the difference between the samples from China and those from Thailand is probably due to the washing process with hot water (*e.g.*, temperature and time) and/or the drying process.

The reason for the unusual value of hypoxanthine in sample No. 3 from Thailand, however, may reflect the freshness of the raw material, assuming these are subjected to the same pretreatment method, because hypoxanthine is the final product of ATP degradation products by various enzymes.[11,12] We compared K' value, which was developed by Saito *et al.*, as an index of the freshness of fish: K value[13] (Table 3).

$$K' \text{ value} = \frac{\text{Hyp}}{\text{IMP} + \text{Ino} + \text{Hyp}} \times 100$$

The K' value obtained from No. 3 was large (81%) compared with other Lumbricus. This indicated that ATP of the samples from China was more decomposed than those from Thailand.

Relatively the same K' values were obtained for other samples (Table 4). The high K' value of Cervi Parvum Cornu, Hippocampus, Kokurozin and Koukuzin may also reflect the length of preservation until pretreatment began

Table 3. Difference of Components and K' Value of Lumbricus

Sample	Location	Nucleic acid components[a]		K' value[b]
		Free	Total	
No. 1	Thailand			10
No. 2	Thailand			7
No. 3	Thailand			81
No. 4	Thailand	18.02±2.68	36.88±2.50	10
No. 5	Thailand			11
No. 6	Thailand			12
No. 7	China			23
No. 8	China	24.00±2.88	34.42±6.06	24
No. 9	China			36

a) Unit: μmol/g dried weight. The data are given as mean±S.D. value of all samples. *b*) Unit: %.

Table 4. K' Value of Animal Crude Drugs

Crude drug[a]	n[b]	Location	K' value[c]
Asini Gelatinum	3	China	83±14
Cicadae Periostracum	3	China	33±58
Amydae Carapax	3	India, *etc.*	93±12
Cervi Parvum Cornu	6	China	71±10
Hippocampus	3	China	94± 2
Kokurozin	3	China	85± 1
Koukuzin	3	China	78± 1

a, b) See Table 1. *c*) See Table 3.

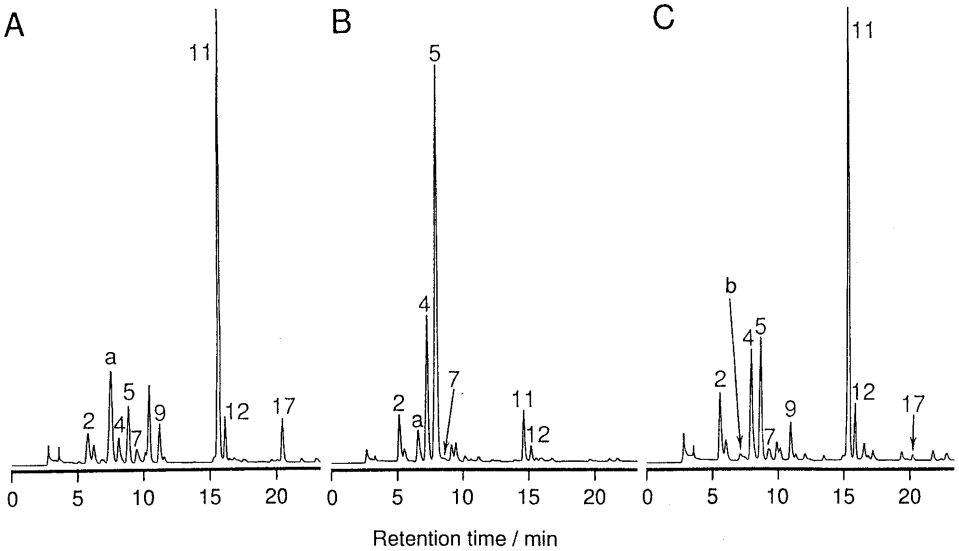

Fig. 2. Assay of the Free Nucleic Acid Components in Three Lumbricus Samples

A: sample No. 1 from Thailand. B: sample No. 3 from Thailand. C: sample No. 8 from China. 2—17: See Fig. 1. a, inosinic acid; b, unknown.

(Table 4).

Conclusion

An analytical method of determining the contents of 7 nucleic acid bases, 6 ribonucleosides and 7 deoxyribonucleosides in animal crude drugs was established. The contents of free nucleic acid components (the contents in ice-cold extracts) and the total contents of nucleic acid components (the contents in heated extracts) in 8 crude drugs showed that each crude drug had a characteristic pattern. It was concluded that the contents free and total nucleic acid components could be useful in the evaluation of each animal crude drug.

If we are able to obtain many samples which are clear in origine, details of the pretreatment process and the preservation conditions used, we would like to confirm the above conclusions by determining the content of free and total nucleic acid components.

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References and Notes

1) A part of this work was presented at the 24th Symposium on Natural Drug Analysis, Osaka, November 1995.
2) Hashimoto A., Yamasaki K., Kokusenya Y., Miyamoto T., Sato T., *Chem. Pharm. Bull.*, **42**, 1636—1641 (1994).
3) Hashimoto A., Yamasaki K., Kokusenya Y., Miyamoto T., Nakai H., Sato T., *Chem. Pharm. Bull.*, **43**, 2195—2199 (1995).
4) Hattori M., Yang X., Kaneko S., Nomura Y., Namba T., *Shoyakugaku Zasshi*, **43**, 173—176 (1989).
5) Wang B., Zhao X., Yang X., Kaneko S., Hattori M., Namba T., Nomura Y., *Wakan Iyaku Gaku Zasshi*, **5**, 116—122 (1988).
6) Yamasaki K., Hashimoto A., Kokusenya Y., Miyamoto T., Matsuo M., Sato T., *Natural Medicines*, **48**, 53—57 (1994).
7) "The Japanese Standard of Pharmaceutical Ingredients 1989," Yakuji Nippo, Ltd., Tokyo, 1989.
8) Shneider W. C., *J. Biol. Chem.*, **161**, 293—303 (1945).
9) "The Pharmacopoeia of Japan," 12th Ed., Tokyo, 1991.
10) Namba T., "Coloured Illustrations of Wakan-Yaku," Vol. II. Hoikusha, Osaka, 1980, pp. 253—255,
11) Saito T., Arai K., *Bull. Jpn. Soc. Sci. Fisheries*, **23**, 265—268 (1957).
12) Spinelli J., Ekulund M., Miyauti D., *J. Food Sci.*, **29**, 710—714 (1964).
13) Saito T., Arai K., Matsuyoshi M., *Bull. Jpn. Soc. Sci. Fisheries*, **24**, 749—750 (1957).