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Analytical Studies on the Active Constituents in Crude Drugs. III.[1] High-Speed Liquid Chromatographic Determination of Ecdysterone and Inokosterone in *Achyranthis* Radix

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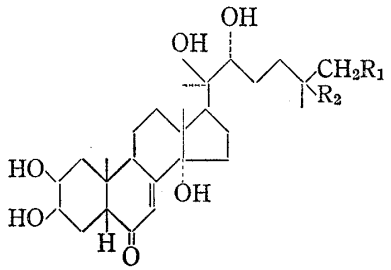
A simple, rapid, and accurate high-speed liquid chromatographic method was established for the determination of ecdysterone (I) and inokosterone (II) in *Achyranthis* radix. I and II, extracted from *Achyranthis* radix, were directly injected onto column and separated in approximately 15 min using a 2 m Permaphase ETH column with a mobile phase of 10% ethanol in *n*-hexane at 50°. A working relative standard deviation was less than 2%. The extraction methods were also investigated and it was concluded that the Soxhlet extraction with methanol was the most effective. Moreover, II was separated into two epimeric isomers on a Permaphase octadecyl silane column with a mobile phase of methanol in water.

Keywords——ecdysterone; inokosterone; high-speed liquid chromatography; *Achyranthis* radix; quantitative analysis; inokosterone isomers; permaphase ETH

Phytoecdysones *e.g.* ecdysterone (I) and inokosterone (II), isolated from *Achyranthis* radix by Takemoto, *et al.*[3] in 1967, showed a high moulting hormone activity in insects or shrimps and high protein anabolic potency in mice.[4–6]

Paper chromatography,[7] thin-layer chromatography,[8] gas chromatography,[9–11] and liquid chromatography[12–14] have been reported for the determination of phytoecdysones, however fewer methods have been described for the separation and the quantitative analysis of I and II in plants.

Takemoto, *et al.*[15] reported the method for the determination of phytoecdysones in *Achyranthis* radix by ultraviolet absorption and fluorometric analysis and



ecdysterone (I) : R_1=H, R_2=OH
inokosterone (II) : R_1=OH, R_2=H

Chart 1

1) Part II: S. Ogawa, A. Yoshida, and Y. Mitani, *Yakugaku Zasshi*, **96**, 1488 (1976).
2) Location: *1-8-1, Tatsuminishi, Ikunoku, Osaka.*
3) T. Takemoto, S. Ogawa, and N. Nishimoto, *Yakugaku Zasshi*, **87**, 325 (1967).
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Jin, *et al.*[16] determined the phytoecdysones in *Achyranthes fauriei* by gas–liquid chromatography (GLC), but either is tedious and time-consuming.

In this report, application of high-speed liquid chromatography (HLC) for the quantitative determination of I and II in *Achyranthis* radix is described. Both were determined simply, rapidly, and accurately by Permaphase ETH column with a mobile phase of 10% EtOH in *n*-hexane. Method of extraction was also studied, to extract I and II from *Achyranthis* radix prior to the measurement by HLC.

In the course of this work, II was separated into C-25 epimers by Permaphase octadecyl silane (ODS) column with a mobile phase of MeOH in water, so the separation of I and these epimers was also examined.

Experimental

Apparatus——A Du Pont liquid chromatograph (Model 830) equipped with a constant temperature air bath, fixed-wave length (254 nm) ultraviolet spectrum (UV) detector, and digital integrator (Shimadzu Model ITG-4A) was used.

Conditions——The analytical conditions were as follows; column: Permaphase ETH, 2.1 m.m.i.d. × 2 m (stainless steel 1 mU tube, Du Pont packed column), column temperature: 50°, mobile phase: 10% EtOH/90% *n*-hexane, flow rate: 0.5 ml/min, sample size: 5 μl, detector sensitivity: 0.04 Aufs.

Reagents and Materials——All solvents used in this study were analytical reagent grade. Ecdysterone and inokosterone were the products of Rohto Pharm. Co., Ltd.

Extraction of Ecdysterone and Inokosterone from *Achyranthis* Radix——Methyl Ethyl Ketone-Extract: (a) To 10 g of finely powdered *Achyranthis* radix (dried over P_2O_5 *in vacuo*), 100 ml of methyl ethyl ketone (MeCOEt) was added and the mixture was refluxed for 1 hr. MeCOEt–layer was transferred and the residue was extracted three times with 100 ml of MeCOEt. The MeCOEt-extracts were combined and evaporated to dryness under reduced pressure.

(b) 10 g of finely powdered dried *Achyranthis* radix was extracted with 200 ml of MeCOEt for 5 hr in Soxhlet extractor. The MeCOEt-extract was evaporated to dryness under reduced pressure.

Methanol-Extract: Two kinds of extracts, MeOH-extract-(a) and (b), were prepared under the same procedure described above.

Preparation of Standard Solution——Ecdysterone: About 40 mg of I standard was weighed accurately into a 200 ml volumetric flask, and diluted to volume with MeOH. Inokosterone: About 20 mg of II standard was weighed accurately into a 200 ml volumetric flask, and diluted to volume with MeOH.

Preparation of Sample Solution——The residues of MeCOEt and MeOH-extracts were dissolved and diluted to 25 ml with MeOH. When these sample solutions gave precipitates, the solutions were centrifuged and the supernatants were used for analysis.

Assay Procedure——5 μl of each standard solution and sample solution was accurately injected by the use of high pressure micro syringe (Hamilton, HP 305) and the area of each peak was determined by digital integrator. The quantity of I or II in each sample solution was calculated by reference to the standard solution.

Calibration Curve——A calibration curve for I or II was constructed by dissolving I or II in MeOH in varying concentration of 0.05—0.4 mg/ml and injecting 5 μl of each standard. The good linearity was established between the concentration of I or II and the peak area or peak hight. A relative standard deviation calculated from six quantitative results in the case of MeOH-extract-(b) gave 1.87%.

Reduction of Sample Solutions with $NaBH_4$——To a 20 ml round bottom flask, 5 ml of each sample solution was transferred, evaporated to dryness under reduced pressure, and 5 ml of water and 300 mg of $NaBH_4$ were added. The mixture was then left standing with occasional shaking for 1 hr, upon which the reduction was completed.

HLC-Conditions for the Separation of Inokosterone Isomers——The operating conditions were as follows; (1) column: Permaphase ODS, 2.1 m.m.i.d. × 1 m (stainless steel 1 mU tube, Du Pont packed column), column temperature: room temp., mobile phase: H_2O→MeOH (2%/min, linear gradient), flow rate: 0.4 ml/min, detector: UV 254 nm. (2) column: Permaphase ODS, 3 m.m.i.d. × 50 cm (glass, dry packed by Umetani autodrypacker), column temperature: room temp., mobile phase: 2% MeOH/H_2O, flow rate: 0.5 ml/min. (3) column: Zorbax ODS, 2.1 m.m.i.d. × 25 cm (stainless steel, Du Pont packed column), column temperature: 40°, mobile phase: 20% MeOH/H_2O, flow rate: 0.4 ml/min.

16) H. Jin, H. Hikino, and T. Takemoto, *Yakugaku Zasshi*, **95**, 596 (1975).

Results and Discussion

The Determination of Ecdysterone and Inokosterone in *Achyranthis* Radix

Chromatographic procedures should be a logical choice for the analysis of phytoecdysones, and of the quantitative chromatographic techniques, GLC is commonly used, but HLC is more attractive than GLC since derivatives are unnecessary and sample preparation may be simple. However, the type of stationary phase employed in HLC is a crucial factor in maximizing these advantages. As a result of investigation on several kinds of stationary phases, normal-phase partition chromatographic technique by Permaphase ETH column was selected for this determination.

Phytoecdysones in *Achyranthes fauriei* were determined by Jin, *et al.*[6] with GLC, but the method to extract them from the plant was not studied sufficiently, so the method of extraction was also investigated.

Four extracts, MeCOEt-extract-(a), (b), MeOH-extract-(a), and (b), were prepared and the contents of I and II were determined by the present method. Chromatograms of the standard and sample solutions are shown in Fig. 1—3 and the quantitative values of I and II in each extract are also shown in Table I. The chromatograms (Fig. 1—3) show the base line separation with I and II on Permaphase ETH column with a mobile phase of 10% EtOH in *n*-hexane at 50°. To confirm whether the peak of I or II was not overlapped with those

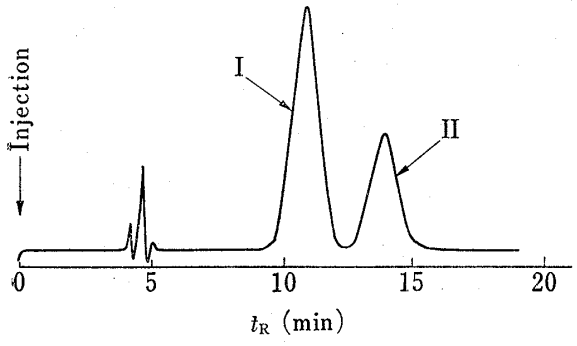

Fig. 1. Chromatogram of Ecdysterone (I) and Inokosterone (II) Standard

conditions: Permaphase ETH (2.1 m.m.i.d. × 2 m), 50°; mobile phase, 10% EtOH/90% *n*-hexane; flow rate, 0.5 ml/min; detector, UV 254 nm; sensitivity, 0.04 Aufs.

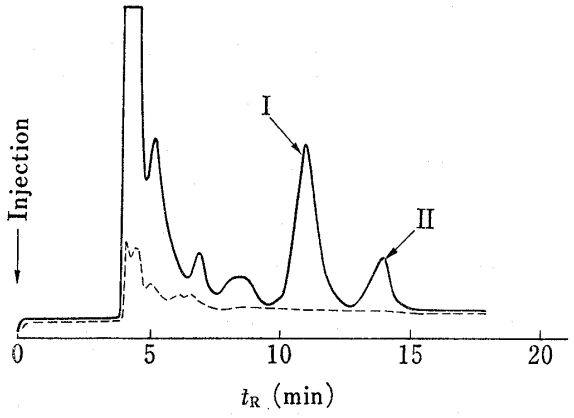

Fig. 2. Chromatograms of I and II in MeCOEt-Extract-(b)

———: sample solution
- - - - - - -: treated with $NaBH_4$
Chromatographic conditions were the same as in Fig. 1.

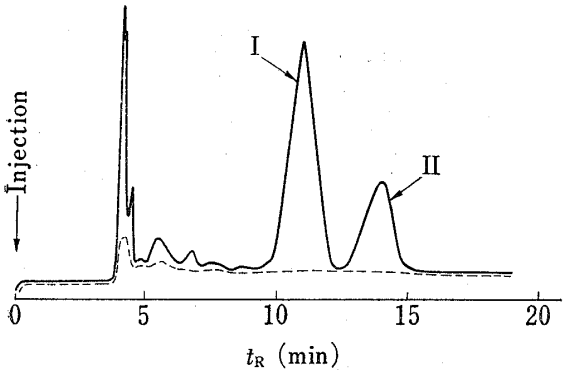

Fig. 3. Chromatograms of I and II in MeOH-Extract-(b)

———: sample solution
- - - - - - -: treated with $NaBH_4$
Chromatographic conditions were the same as in Fig. 1.

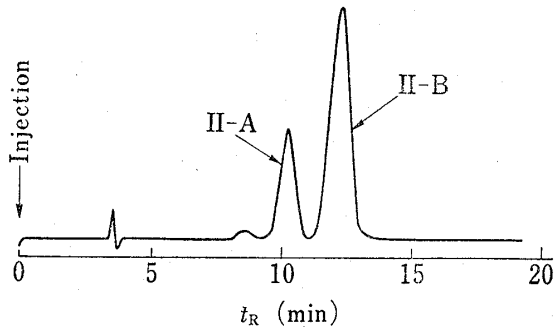

Fig. 4. Chromatogram of Inokosterone Isomers

II-A: inokotesrone A II-B: inokosterone B
conditions: Permaphase ODS (2.1 m.m.i.d. × 1 m), room temp.; mobile phase, $H_2O \xrightarrow{\text{linear-gradients}}$ MeOH (2%/min); flow rate, 0.4 ml/min; detector, UV 254 nm

of other substances in the extracts, the sample solutions were treated with $NaBH_4$ and chromatographed under the same conditions. The dotted lines in Fig. 2—3 show the chromatograms of the reduced sample solutions demonstrating that interfering substances do not exist. On the other hand, when the MeOH-extract-(b) was chromatographed on a silica gel thin-layer plate, and the separated phytoecdysones were analyzed by this HLC, almost same analytical values were obtained. And when the method described by Takemoto, *et al.*[15] was applied for the determination of I and II in the MeOH-extract-(b), similar values were obtained. These facts suggest that phytoecdysones in *Achyranthis* radix can be determined by the present HLC method without influence of interfering substances.

Table I. The Content and the Ratio of Ecdysterone and Inokosterone in *Achyranthis* Radix

Sample solution	Content (%)		Ratio (Ecd./Inoko.)
	Ecdysterone	Inokosterone	
MeCOEt-Extract-(a)	0.008	0.004	66/34
MeCOEt-Extract-(b)	0.027	0.014	66/34
MeOH-Extract-(a)	0.065	0.032	67/33
MeOH-Extract-(b)	0.069	0.037	65/35

Table I shows the contents and the ratio of I to II in each extract, and suggests followings: 1. The MeOH-extract-(a) and (b) give higher phytoecdysone content than the MeCOEt-extracts. 2. The extraction rate of phytoecdysones with Soxhlet extractor is better than refluxing in the case of MeCOEt-extract, but a significant difference is not perceived between MeOH-extract-(a) and (b). Comparing MeOH-extract-(a) with (b), (b) is better than (a), because it is prepared easily and simply. 3. The ratio of I to II gives about 66: 34 in each extract, and the difference by means of extraction solvents or methods are not recognized. Consequently, for the extraction of phytoecdysones from *Achyranthis* radix, Soxhlet extraction with MeOH seems to be the best.

The Separation of Inokosterone Isomers

In the course of this work, II was separated into two components (Fig. 4). II is expected to be a mixture of epimeric isomers of C-25, but these isomers have never been separated and identified.

Under the optimum conditions shown in Fig. 4, two isomers were able to be made base line separation. Components corresponding to these two peaks were isolated by the preparative HLC with Permaphase ODS (16.7 m.m.i.d. × 50 cm) column, then nuclear magnetic resonance spectrum, infrared spectrum, or mass spectrum were measured. The results suggested that these were epimeric isomers of C-25 and the detailed results will be reported later. We named one of two isomers inokosterone A (II-A), which was eluted faster from Permaphase ODS column, and the other isomer inokosterone B (II-B).

The ratio of II-A to II-B was about 1.7 in crystallines obtained from *Achyranthis* radix, but in case of other plants gave different values. The crystalline isolated from *Woodwardia orientalis* showed the highest ratio *i.e.* 2.8.

Attempts to separate the three phytoecdysones, I, II-A and II-B, are unsuccessful because of the incomplete separation between I and II-A, especially when the quantity of I is more than II. However, when the quantity of I is less than II, three compounds can be almost separated.

II-A and II-B can also be separated on Zorbax ODS (2.1 m.m.i.d. × 25 cm) or Permaphase ODS (3.0 m.m.i.d. × 50 cm) column with a mobile phase of MeOH in water with constant elution, but separation of three phytoecdysones is unsuccessful.

Present method provides a simple, rapid and accurate tool for the separation and quantitative determination of I and II in *Achyranthis* radix. In addition, II is separated into two epimeric isomers by Permaphase ODS or Zorbax ODS column with a mobile phase of MeOH in water. This HLC method will be also applied to the purity measurement of I or II.

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