Agric. Chem. Biotechnol. **41**(4), 251-256 (1998)

Article

Development of Analytical Methods for Insect Moulting Hormone (β-Ecdysone) by HPLC/UV Using Boronate Derivatization

Jae-Han Shim*, In-Seon Kim and Kye-Taek Lim[1]

Department of Agricultural Chemistry, Chonnam National University, Kwangju 500-757, Korea
[1]Division of Protein Engineering, Institute of Biotechnology, Chonnam National University, Kwangju 500-757, Korea

Received May 16, 1998

The analytical method of β-ecdysone, the insect moulting hormone, by high performance liquid chromatograph (HPLC) with UV detector was developed using boronic ester derivatization and applied to the extracts of *Ajuga iva, Silene otites* and *Schistocerca* egg. Derivatization of yield with methyl-, butyl-, and phenyl-boronate was completed under mild conditions with 20-hydroxyecdysone. The conversion ratios of boronate were estimated to be 70% in methylboronic acid, 89% in butylboronic acid and 93% in phenylboronic acid. Phenylboronate showed a high sensitivity and demonstrated an effective separation on HPLC. The optimum temperature and reaction time for derivative formation were 25°C and 20 min. respectively. β-Ecdysone was effectively identified in extracts of *Ajuga iva, Silene otites* and *Schistocerca* egg by the HPLC method.

Key words: *boronic ester derivatization, insect moulting hormone, 20-hydroxyecdysone.*

Ecdysteroids are significant natural compounds controlling the mechanism of moulting and metamorphosis of arthropods and are derived from the insect moulting hormone ecdysone. They are widely distributed in both plants and invertebrates. They regulate a series of important physiological functions, mostly in insects and in other arthropods.[1)] However, their function in plants is still unclear.[2,3)] Almost 200 kinds of structural analogues have been isolated from plant and animal sources so far.[4)] They occur predominantly in biological materials in complex mixture. In plants, they are often represented as one or two major constituents with admixtures of various minor structurally and biogenetically relative substances.

The ecdysteroids are large families of polyhydroxy steroids, structurally related to ecdysone. They are usually in mono hydroxylated or glycoside form. They are nearly always existent in complex mixtures of one or two main constituents and of several additional minor derivatives or analogues. Their important structural feature is the presence of α, β unsaturated carbonyl group in ring B (λ_{max}=243 nm) that allows the UV detection in HPLC analysis. Derivatization techniques which have been reported for ecdysteroids are therefore concentrated on changing spectral and chromatographic properties. Phenanthrene boronic acid has been used as fluorescence label in the TLC of ecdysteroids,[5)] and butylboronic acid along with phenyl- and 3-aminophenylboronic acids have been used as mobile phase additives or derivatization agents in TLC of certain ecdysteroids.[6)] Arylboronic acid bound to the silica gel has been proposed as a selective sorbent for solid phase extraction (SPE) of ecdysteroids.[7)] An application of alkyl and arylboronic acids in supercritical fluid chromatography (SFC) of ecdysteroids has been reported.[8)] Phenylboronic acid also has been used as a derivatization agent for HPLC analysis. Extensive data on chromatographic determination of ecdysteroids have been known.[9-12)]

However, as a result of constantly growing number of known ecdysteroids and rather subtle structural differences occurring within ecdysteroid family, the separation of some compounds may still be a problem. It is therefore important to have suitable, simple, and specific methods for the rapid detection and identification of ecdysteroids in complex mixtures.

The aim of this paper is to demonstrate the possibility of using derivatization with a few alkyl and aryl boronic acids for reversed phase HPLC of ecdysteroids and to study the capability of formed ecdysteroid boronates to improve the separation efficiency of hardly separable ecdysteroids.

*Corresponding author
Phone: 82-62-530-2135; Fax: 82-62-530-2139
E-mail: jhshim@orion.chonnam.ac.kr

Materials and Methods

Chemicals. Authentic standards of 20-hydroxyecdysone (I), 2-deoxy-20-hydroxyecdysone, polypodine B, and ecdysone were purchased from Sigma (St. Louis, MO, U.S.A) and the compounds of methyl-, butyl-, and phenyl-boronic acid were purchased from Aldrich (Milwaukee, WI, U.S.A). These chemicals were analysed to examine the purity by HPLC prior to use.

HPLC analysis. HPLC measurements were performed using a Waters Model 510 pump and Model 486 UV detector. A Waters 3.9 μm×300 mm RP C-18 Bondapak Stainless Column was used. The eluent system was 87% MeOH in H_2O at a flow rate of 1.0 ml/min. Samples (10 μl) were injected into the system through a Rheodyne injection valve with a 20 μl injection loop. UV detection was set at 240 nm to monitor.

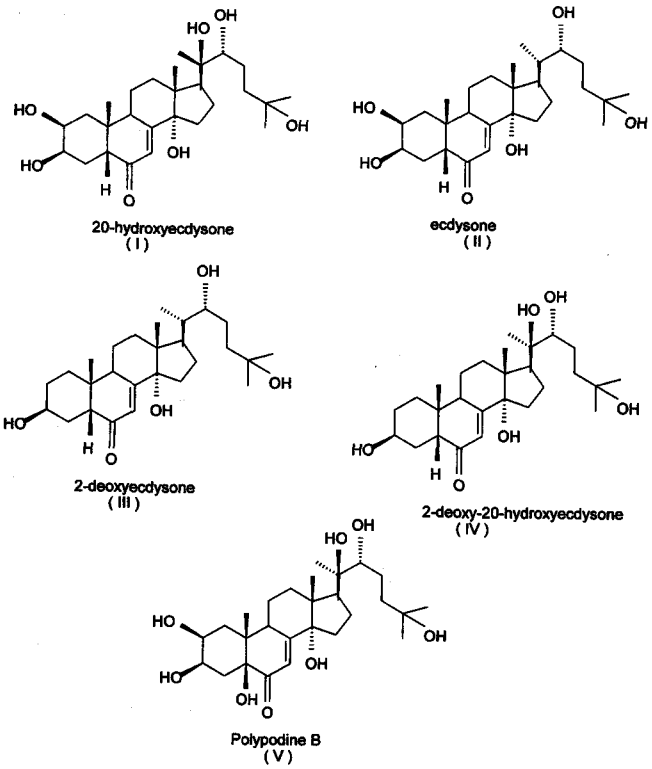
The samples of plant extracts of *Silene otites, Ajuga iva*, and *Schistocerca* eggs were given by R. Lafond. Ecdysteroids and boronic acids were prepared in dilute solutions with methanol. Varying amounts of methyl-, butyl-, and phenyl-boronic acid were added to methanol solution of ecdysteroid and the reaction mixture was left for 20 min at room temperature before chromatography.

Formation of boronic esters of ecdysteroids. The formation of boronic ester was accomplished by the reaction of 20-hydroxyecdysone with three representative boronic acids (Fig. 1). Methyl-, butyl-, and phenyl-boronic acid were dissolved in MeOH to give a concentration of 0.25 to 500 mM. The aliquot of boronic acid was added to the solution of 20-hydroxyecdysone (0.2 μM) and the final volume was adjusted to 100 μl with the fresh methanol. The reaction mixture was stirred for 5 min. at 25°C and the solvent was removed by gentle nitrogen stream. Plant extracts (*Silene otites* and *Ajuga iva*) and *Schistocerca* eggs were dried and dissolved in an appropriate amount of methanol and sonicated for 2 min. The aliquot was reacted as described above.

Determination of boronic ester of ecdysteroids. The determination of boronic ester was conducted by RP C-18 column HPLC equipped with UV detector. The dried sample of boronic ester was dissolved in an appropriate amount of methanol and 10 μl of the sample was subjected to HPLC. The ratio of boronic ester was calculated by comparing boronic acid with ecdysone-boronate.

Results

Formation of boronic ester of 20-hydroxyecdysone. The conversion ratio of boronic ester were 70% in methyl-, 89% in butyl-, and 93% in phenyl-boronic acid. The optimum condition of temperature and reaction time for the formation of boronic ester was 25°C and 20 min, respectively (Table 1).



Chemical structures of ecdysteroids

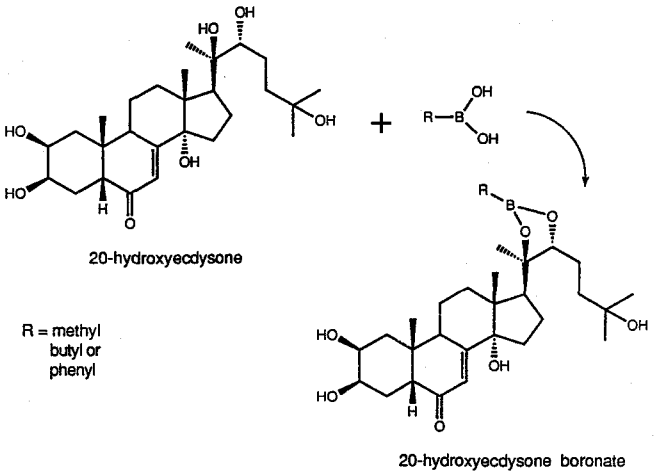


Fig. 1. Formation of a cyclic bornic ester of 20-hydroxyecdysone.

HPLC analysis of boronic ester of 20-hydroxyecdysone. The HPLC chromatograms of boronic acid and its ester compounds were shown in Fig. 2, 3 and 4. The formation of the phenylboronic ester was accomplished much more readily than that of either the methyl or butyl esters which required seven-fold greater excess of reagent for complete conversion. The electron-donating effect of the phenyl ring evidently assisted ester formation. Conversion to a particular boronic ester had different effects upon ecdysteroid. The retention time of methylboronic ester was 5.3 min, butylboronic ester, 13.2 min, and phenylboronic ester 11.3 min. The methyl ester had the shortest retention while the butyl ester had the

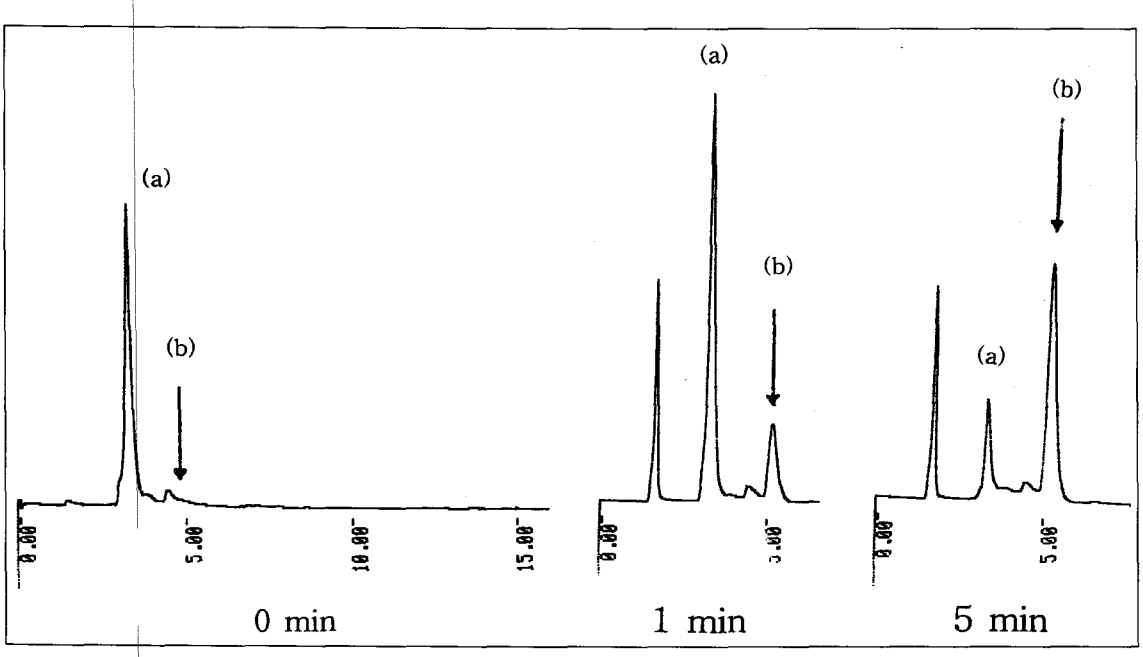


Fig. 2. HPLC chromatograms of 20-hydroxyecdysone after reaction with 0.25 mM of methylboronic acid for 1 and 5 min at room temperature. (a) 20-hydroxyecdysone 90.2 μM), (b) 20-hydroxyecdysone methylboronate.

Table 1. Effect of time and temperature for the esterification reaction between 20-hydroxyecdysone (0.2 μM) and various boronic acid in MeOH

Boronic acid	Molar excess (mM)	Temperature (°C)	Time (min)	Yield of boronic ester (%)
Methyl	0.25	25	1	14
		25	5	62
		25	15	68
		25	20	70
		50	3	56
Phenyl	0.25	25	1	33
		25	5	63
		25	15	88
		25	20	93
		50	3	85
Butyl	0.25	25	1	11
		25	5	62
		25	15	80
		25	20	89
		50	3	60

longest to the parent ecdysteroid.

Determination of boronic ester. Plots of the extent conversion to boronic ester against quantity of boronic acid added were given in Fig. 5. The temperature for the reaction had little effect on the reaction. To test the use of these boronic esters in natural materials, we had examined crude extracts of desert locust (*Schistocerca gregaria*) eggs, which contained ecdysone (II) and 2-deoxyecdysone (III) after enzymic hydrolysis, and extracts of *Ajuga iva* which contained 20-hydroxyecdysone (I), 2-deoxy-20-hydroxyecdysone (IV) and polypodine B (V) and an extract of *Silene otites* which contained polypodine B (V) and 20-hydroxyecdysone (I).

The *Schistocerca* eggs contained ecdysteroids which did not form boronic esters. However, the main peaks were unaffected by addition of boronic acid and small amount of complex forming ecdysteroid in the mixture (Fig. 6). In the case of the *Ajuga* and *Silene plant* extracts, all the components were shifted to the longer retention time area when sufficient boronic acid was added (Fig. 7, 8). It also showed that all the components in the extracts contained the 20,22-diol structure, although compounds I", IV" and V" showed one unresolved peak as their boronic esters did, under the same conditions.

Discussion

The isolation of ecdysteroids from biological materials needed a skillful and lengthy process. Developing effective way for the isolation, and utilizing special properties of ecdysteroids, would be a considerable help in identification and quantitative analysis. One possible strategy would be a exploit the existence of vicinal diol systems (C-2 and C-3, or at C-20 and C-22, or both) found in many, if not in most ecdysteroids.

The experiments with model ecdysteroids showed that there were different ways for boronic ester formation between the C-20,22 diols and the C-2,3 diols. All of 20-hydroxyecdysone (I), 2-deoxy-20-hydroxyecdysone (IV), and polypodine B (V) possessed C-20,22 diols and all formed cyclic boronates. Ecdysone (II), which possessed C-2,3 diols but not C-20,22 diols, showed no evidence of cyclic ester formation under mild conditions even with a large excess of reagent. This was consistent with the result of a study on the use of immobilized boronic acids

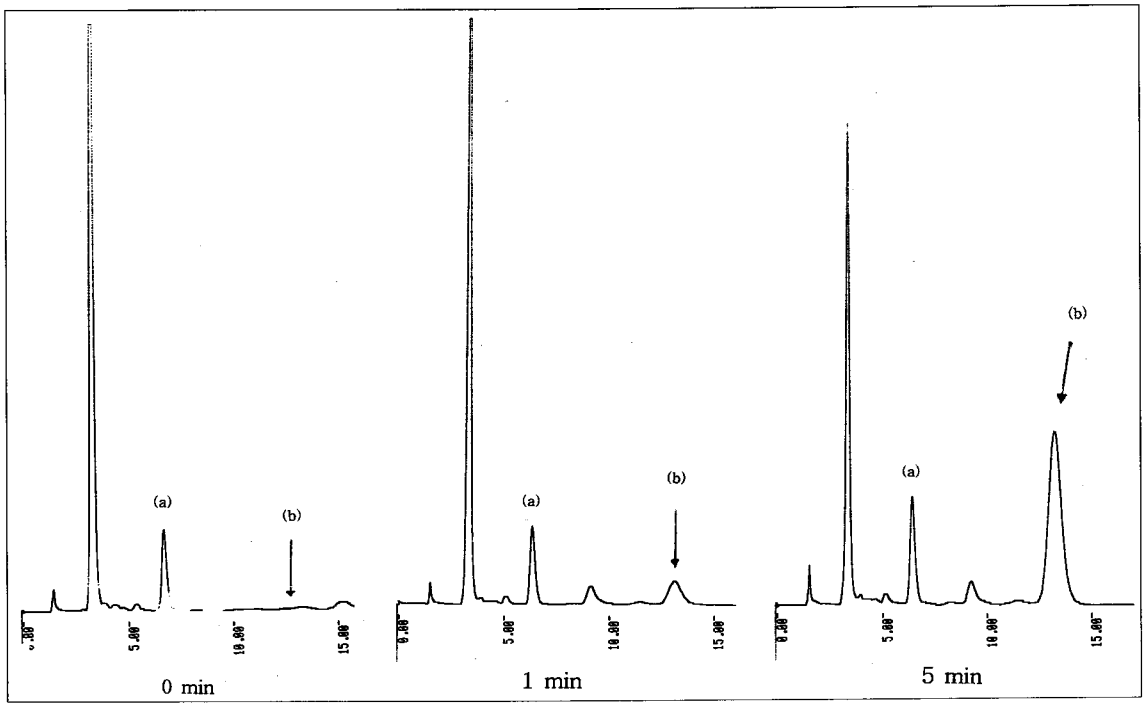


Fig. 3. HPLC chromatograms of 20-hydroxyecdysone after reaction with 0.25 mM of butylboronic acid for 1 and 5 min at room temperature. (a) 20-hydroxyecdysone (0.2 μM), (b) 20-hydroxyecdysone butylboronate.

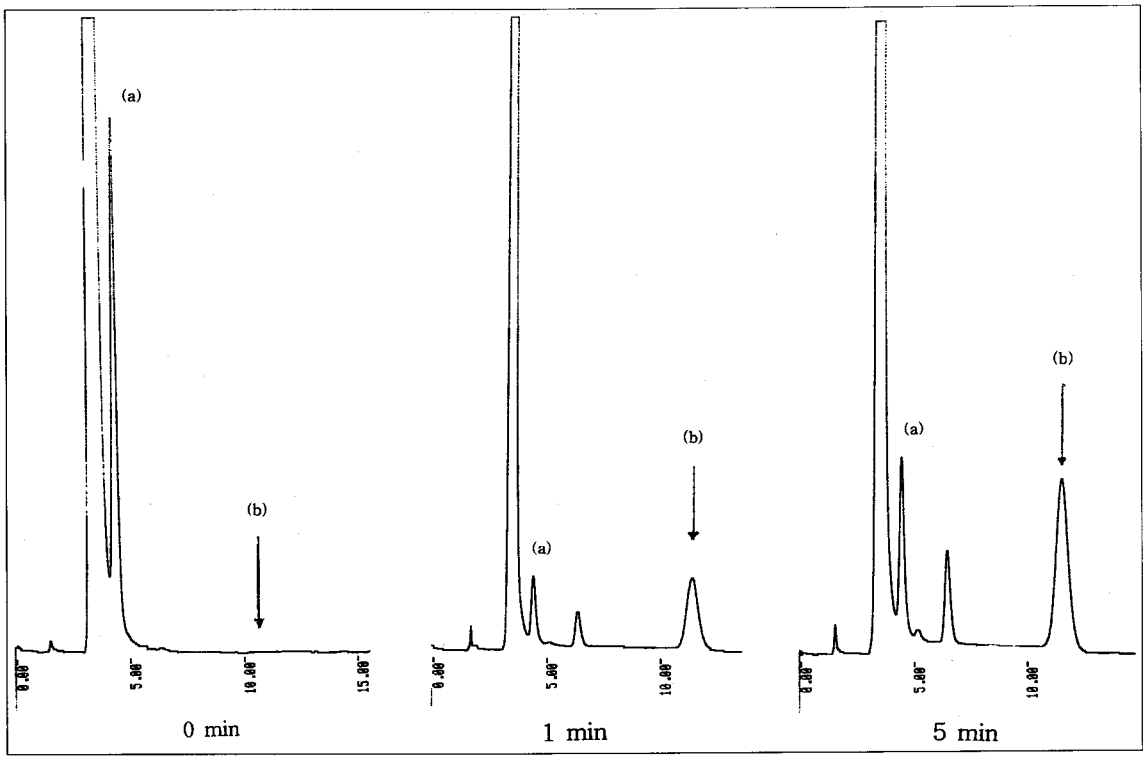


Fig. 4. HPLC chromatograms of 20-hydroxyecdysone after reaction with 0.25 mM of phenylboronic acid for 1 and 5 min at room temperature. (a) 20-hydroxyecdysone (0.2 μM), (b) 20-hydroxyecdysone phenylboronate.

for solid-phase ecdysteroids.[6] Ecdysteroid with C-2,3 cis diols did not make cyclic boronates under the conditions explored.

As the C-2,3 diol moiety was not able to form stable boronates, these compounds could not be determined by this method. The method was limited to the major group of 20-hydroxyecdysteroids.[11]

Addition of an excess of 1% solution of methyl-, butyl-,

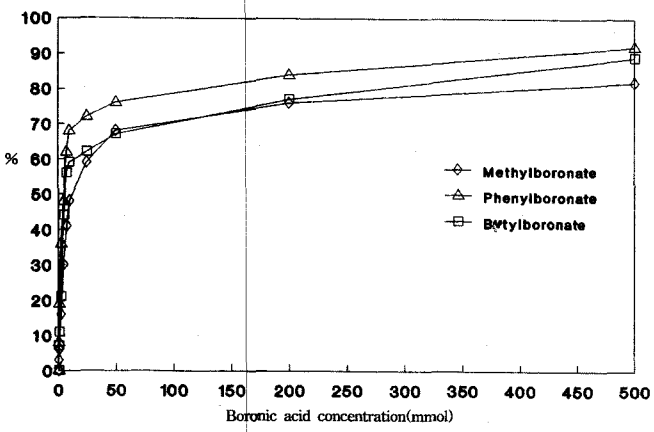


Fig. 5. The formation rate of boronic ester of 20-hydroxyecdysone (0.2 μM) with various amounts of boronic acid.

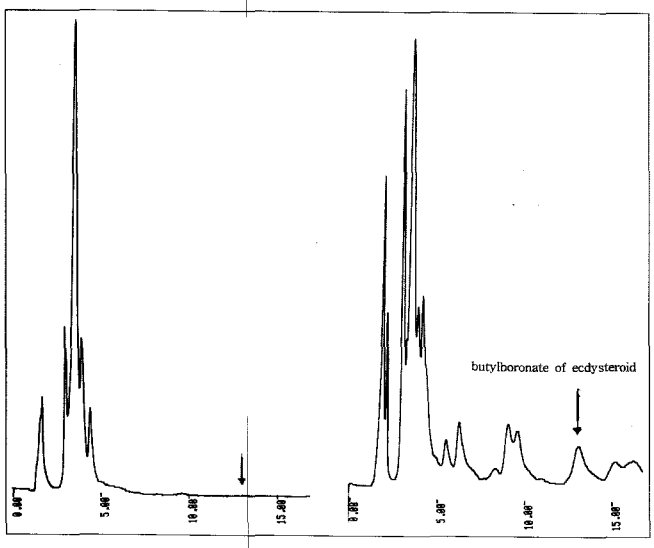


Fig. 6. HPLC chromatograms of *Schistocerca* eggs extract before (left) and after (right) treatment with butylboronic acid.

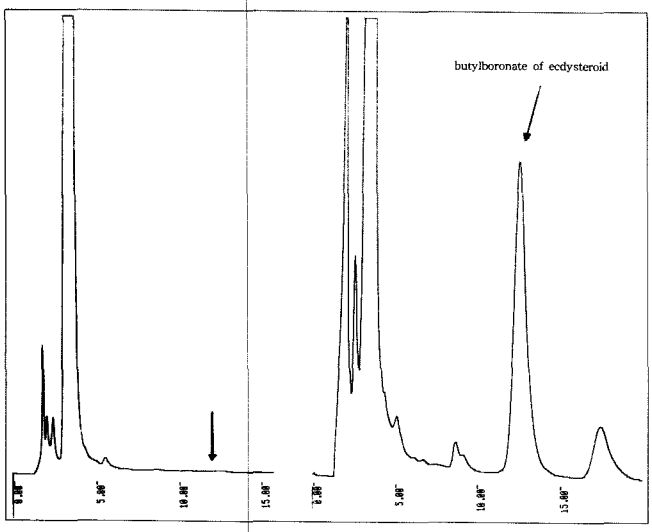


Fig. 7. HPLC chromatograms of *Ajuga iva* extract before (left) and after (right) treatment with butylboronic acid.

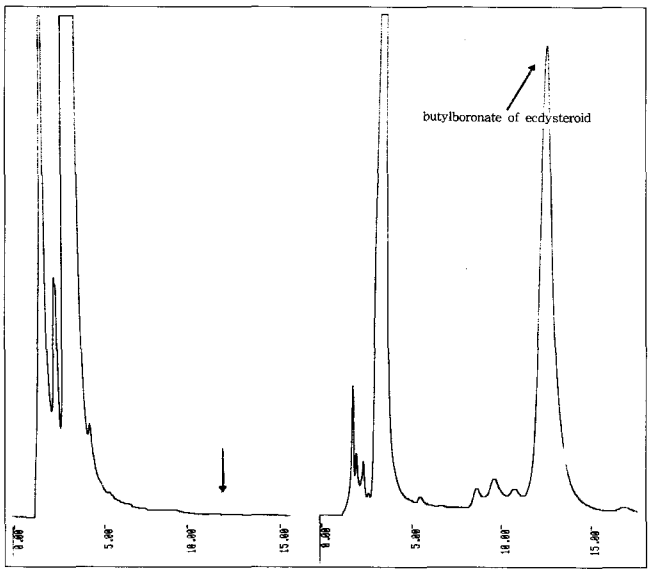


Fig. 8. HPLC chromatograms of *Silene otites* plant extract before (left) and after (right) treatment with butylboronic acid.

or phenyl-boronic acid to dilute (2 μM) solution of ecdysteroid possessing a C-20,22 diol moiety resulted in a rapid formation of stable cyclic boronate ester which had a longer retention time than the parent ecdysteroid.

These experiments demonstrated that boronic esters could be formed in natural conditions, without using catalysts and with impure mixtures. These less polar esters could give a rapid indication of C-20,22 diol ecdysteroids in crude plant or animal extracts and could be a useful guide for the screening of plant materials. The phenylboronic esters was preferred for a rapid formation. But the butyl boronic ester was recommended, if a larger shift in reaction was required.

The boronic ester of 20,22-dihydroxyecdysteroids could be prepared by simple mixing the methanolic solutions of samples and appropriate boronic acid. The reaction was usually completed within 20 min and it could be carried out both with pure ecdysteroids and with crude plant and insect extracts. The prepared boronates showed sufficient stability under the condition of RP-HPLC. This derivatization reaction could be employed as a simple and direct way to C-20,22 diol group in ecdysteroid molecule. Changed chromatographic behavior of prepared derivatives could significantly contribute to HPLC determination of compounds which were hardly separable.

Acknowledgments. This work was supported by the Korea Science and Engineering Foundation (951-0604-038-2).

References

1. Morgan, E. D. and Wilson, I. D. (1989) Methods for

separation and physico-chemical quantifiction of ecdysteroids. In *Ecdysone*, Koolman, J. (ed.) pp. 114-130, Georg Thieme-Verlag, Stuttgart.

2. Lafont, R., Bouthier, A. and Wilson, I. D. (1991) Insect chemical ecology, In *Proceeding of the conference on insect chemical ecology*, pp. 197-207, SPB Academic Publishing, The Hague.
3. Camps, F. (1991) In *Ecological chemistry and biochemistry of plant terpenoids*, Harborne, J. B. and Tomas-Barberan, F. A. (eds.) pp. 331-345, Clarenden Press, Oxford.
4. Lafont, R. and Wilson, I. D. (1992) In *The ecdysone handbook*, Chromatographic Society, Nottingham.
5. Poole, C. F., Singhawangcha, S., Zlatkis, A. and Morgan, E. D. (1978) Polynuclear aromatic boronic acid as selective fluorescent reagents for HPTLC and HPLC. *J. High Resol. Chromatog. Chromatog. Commun.* **1**, 96-97.
6. Wilson, I. D. (1992) The use of boronic acids for the normal and reversed-phase TLC of Ecdysteroids. *J. Planar Chromatogr.* **5**, 316-320.
7. Murphy, S. J., Morgan, E. D. and Wilson, I. D. (1990) In *Chromatography and isolation of insect hormones and pheromones*, McCaffery, A. R. and Wilson, I. D. (eds.) pp. 131-145, Plenum Press, New York.
8. Shim, J. H., Wilson, I. D. and Morgan, E. D. (1993) Boronic esters as derivatives for supercritical fluid chromatography of ecdysteroids. *J. Chromatogr.* **639**, 281-285.
9. Wilson, I. D., Bielby, C. R. and Morgan, E. D. (1982) Evaluation of some phytoecysteroids as internal standards for the chromatographic analysis of ecdysone and 20-hydroxyecdysone from arthropods. *J. Chromatogr.* **236**, 224-229.
10. Marco, M. P., Sanchez-Baeza, F. J., Camps, F. and Coll, J. (1993) Phytoecdysteroid analysis by high-performance liquid chromatography-thermospray mass spectrometry. *J. Chromatogr.* **641**, 81-87.
11. Lafont, R., Morgan, E. D. and Wilson, I. D. (1994) Chromatographic procedures for phytoecdysteroids. *J. Chromatogr.* **658**, 31-53.
12. Pis, J. and Harmatha, J. (1992) Phenylboronic acid as a versatile derivatization agent for chromatography of ecdysteroids. *J. Chromatogr.* **596**, 271-275.