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Review

Liquid chromatographic assay of brassinosteroids in plants

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ABSTRACT

The liquid chromatographic assay of brassinosteroids (BRs) in plants as boronate derivatives with UV, fluorimetric and electrochemical detection is described. Several boronic acid derivatives proved to be satisfactory for use in the derivatization of BRs with respect to reactivity and sensitivity. BRs were readily condensed with the boronic acid derivatives under mild conditions to provide the corresponding boronates, which exhibit strong absorption, fluorescence or maximum amperometric sensitivity with detection limits of *ca.* 20–100 pg, depending on the prelabelling reagents. The method was successfully applied to the determination of natural BRs. BRs were identified in the pollen of broad bean (*Vicia faba* L.), sunflower (*Helianthus annuus* L.) and buckwheat (*Fagopyrum esculentum* Moench). The results demonstrate that the liquid chromatographic microanalytical method is useful for the screening of BRs in the plant kingdom.

CONTENTS

1. INTRODUCTION

Since the discovery of brassinolide (BL) in 1979 [1], studies on BL and its related steroids (brassinosteroids, BRs) have greatly widened our knowledge of this class of steroidal plant hormone. It has been found that BRs show a wide range of biological activities when compared with other known phytohormones, and

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that BRs promote plant growth and increase crop yields [2]. Intensive and extensive studies on the isolation of new BRs from plant sources and on their screening in plants have been carried out, mainly in Japan. It has been found that a number of BRs occur in a wide variety of higher plants and also in some lower plants [2].

Because of their high biological activity and their low concentrations in plants, bioassays to guide fractionation during purification steps and microanalytical methods are necessary for the screening and identification of BRs in plants. A rice-lamina inclination test [3] and microanalysis by gas chromatography–mass spectrometry (GC–MS) [4–7] have been very effective in these studies. In fractionating and isolating very small amounts of BRs from plants, sensitive and specific bioassays for BRs are indispensable. A bean-second internode assay was used to isolate BL from rape (*Brassica napus* L.) pollen [1], and the rice-lamina inclination test was used to isolate castasterone (CS) from chestnut (*Castanea* spp.) insect galls [8]. Subsequently, the latter bioassay has been widely employed to isolate many BRs from a number of plant sources, because of its simplicity, high sensitivity and specificity for BRs.

Isolation of BRs in pure form is a time-consuming and tedious task because of their very low concentrations in plants. Therefore, in order to determine BRs in a partially purified fraction, they should be converted into suitable derivatives. In the GC–MS microanalysis method, natural BRs have been measured as their bismethaneboronate or methaneboronate–trimethylsilyl derivatives [4–7]. GC–MS analysis has contributed to the identification and characterization of more than 30 natural BRs in many higher plants and some lower plants [7]. Among the natural BRs, CS occurs most frequently, followed by BL, suggesting that these two BRs are important in plant growth and development. With respect to the content of BRs, pollen is the richest source. Immature seeds also have a high content of BRs, whereas shoots and leaves have lower levels [2].

High-performance liquid chromatography (HPLC) is a useful tool for the separation and determination of trace amounts of naturally occurring compounds. The study of micro-scale analyses for BRs includes some basic problems: (1) highly sensitive methods of detection are required because the levels of BRs in plants are the lowest among the known phytohormones; and (2) highly selective separation methods are needed because several analogues of BRs coexist in plants. These two problems must be solved and the highest level of analytical sensitivity must be achieved.

In HPLC, UV–Vis, fluorimetric and electrochemical detection methods are commonly used to achieve the highly sensitive and selective determination of many kinds of analytes. Prelabelling derivatization methods in HPLC are frequently used to enhance the sensitivity and selectivity of detection of compounds that do not have a suitable chromophore, fluorophore or electrophoric group in the molecules. Because of the lack of a UV-active chromophore or a fluorophore in BRs, derivatization is essential for their LC microanalysis. BRs could be derivatized to highly detectable derivatives because they usually have four hydroxyl groups as two sets of vicinal diols, in the A-ring ($2\alpha,3\alpha$-position) and in the side-chain ($22R,23R$-position).

Since 1988, we have been developing the liquid chromatographic assay of natural BRs according to the prelabelling method as their boronate derivatives [9]. In this review we describe several kinds of boronic acid derivatives as prelabelling reagents for BRs (Fig. 1), and we also demonstrate the potential of the HPLC method by the separation of standard BRs and its application of the determination of natural BRs in some pollens.

2. DERIVATIZATION

Poor selectivity was observed in the prelabelling of four hydroxyl groups of BRs by naphthoyl chloride [10] or anthroyl nitrile [11], which are excellent prelabelling reagents for hydroxyl groups, because the derivatives afforded complex peak patterns in the liquid chromatograms under reversed-phase conditions.

Boronic acid derivatives give high selectivity and reactivity for the *cis*-1,2-diols of BRs. The

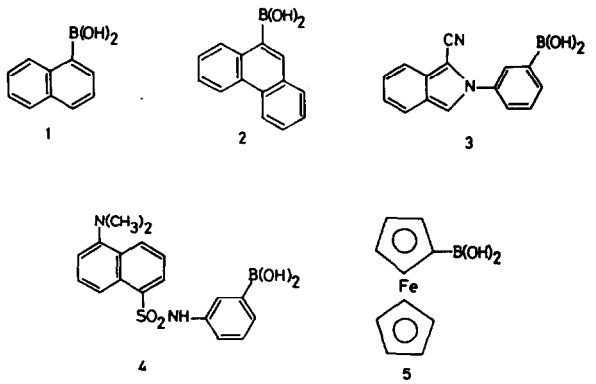

Fig. 1. Boronic acid derivatives for derivatization of brassinosteroids. 1 = Naphthaleneboronic acid; 2 = phenanthreneboronic acid; 3 = 1-cyanoisoindole-2-*m*-phenylboronic acid; 4 = dansylaminophenylboronic acid; 5 = ferroceneboronic acid.

design of a derivatization reagent for the LC separation of BRs requires two structural features: a functional group highly reactive towards the 1,2-diol group and a very responsive fluorophore or electrophore for detection. For this purpose, we synthesized 1-cyanoisoindole-2-*m*-phenylboronic acid and dansylaminophenylboronic acid. Information on early developments in the formation of cyclic boronates was reported in general reviews on cyclic derivatives of bifunctional compounds by Poole and Zlatkis [12].

Previous derivatizations using methaneboronic acid in GC analysis have usually been carried out under dry pyridine. After investigation of the reaction conditions, it was found that a catalytic amount of pyridine was essential to achieve boronate formation. Accordingly, the optimum reaction conditions were as follows: a standard mixture or a biologically active BR fraction was dissolved in a small amount of acetonitrile (100 μl), 100 μl of boronic acid derivatives (1 mg/ml) in 1% (v/v) pyridine–acetonitrile were added (an excess of the reagents over BRs) and the mixture was heated at 70°C for 10–20 min. The general scheme of boronate formation using BL as a substrate is illustrated in Fig. 2. Different reactivities between the boronic acid derivatives has not been observed. The boronate derivatives formed have been found to be stable for at least 2–3 months in a freezer.

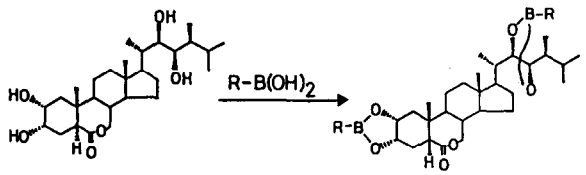

Fig. 2. General scheme for the formation of brassinolide boronates using boronic acid derivatives [R–B(OH)$_2$].

3. SEPARATION MODE

In the LC analysis of steroid compounds, adsorptive separation using silica or reversed-phase separation using an ODS- or CN-bonded column has generally been selected. We selected the reversed-phase mode for the analysis of BRs. The main reason was that reversed-phase columns, especially ODS columns, have good stability and durability, and a wide range of mobile phases are available for the separation of analytes.

A comparison of a number of ODS columns revealed that some of them showed peak tailing in the chromatograms of the series of 6-ketone compounds (*e.g.*, CS). This reflected the content of silanol groups remaining on the surface of ODS-derivatized silica. Although many studies have been carried out to produce highly efficient ODS columns, the problems of end-capping treatment of the silanol on ODS-silica still remains. Throughout this work we selected ODS columns whose silanol groups are sufficiently excluded from the ODS-silica. Attempted separations of BRs using C_8- or CN-bonded columns failed to give good separation patterns on the chromatograms.

4. UV DETECTION

There has been no report of the application of naphthaleneboronic acid as a derivatization reagent for HPLC. We examined the derivatization of BRs with this reagent [13]. The electron impact (EI) mass spectrum of the boronate of BL afforded a molecular ion at *m/z* 752, which confirmed the formation of the bis-naphthaleneboronate. The other BRs were also derivatized to the corresponding bisboronates.

TABLE 1

RETENTION TIMES OF NAPHTHALENEBORONATES OF BRASSINOSTEROIDS

HPLC conditions: Shim-pack CLC-ODS column (15 cm × 6.0 mm I.D.); mobile phase, acetonitrile–water (75:25, v/v); flow-rate, 1.2 ml/min; temperature, 45°C; detection, 280 nm.

Compound	t_R (min)
28-Norbrassinolide	6.24
Dolichosterone	7.87
28-Norcastasterone	8.14
Brassinolide	8.54
Castasterone	11.66
28-Homobrassinolide	11.81
28-Homocastasterone	16.68

Separation of these boronates was examined using a conventional reversed-phase ODS column. When a Shim-pack CLC-ODS column was used at 45°C with acetonitrile–water (75:25, v/v) as the mobile phase, the boronates of BL and its side-chain analogues were detected at 280 nm and exhibited sharp peaks. The retention times of the boronates of seven authentic BRs are given in Table 1.

A narrow-bore column such as a Shim-pack SBC-ODS afforded better resolution, sharper peaks and shorter separation times. We examined the detection limit of the BL naphathaleneboronate using the column. The result showed that BL can be detected down to at least 100 pg per injection (signal-to-noise ratio = 2) as its boronate derivative.

5. FLUORIMETRIC DETECTION

As a continuation of our studies on highly sensitive detection methods for BRs, we screened fluorescent boronic acid derivatives as derivatization reagents in the hope of developing a more sensitive and selective method.

First, we examined the prelabelling derivatization of BRs with phenanthreneboronic acid (commercially available) [14]. Although Poole *et al.* [15] reported its application as a derivation reagent to detect ecdysteroids by TLC, no data were available for its use in HPLC. The EI mass spectrum of the boronate derivative of BL confirmed the formation of the bisphenanthreneboronate.

For the separation of the phenanthreneboronates of BRs, an STR ODS-H column was found to afford better resolution than several conventional ODS columns tested. We examined the detection limits of these PBs using this column and acetonitrile–water as the mobile phase. The boronates were monitored at an emission wavelength of 375 nm when excited at 305 nm. As shown in Fig. 3, the phenanthreneboronates of seven BRs afforded sharp peaks. The method gave a detection limit for BL 50 pg per injection (signal-to-noise ratio = 3). A fivefold increase in detectability was obtained in comparison with UV absorption detection of the naphthaleneboronates.

Second, we examined 1-cyanoisoindole-2-*m*-phenylboronic acid, which is readily prepared from *o*-phthalaldehyde (OPA) and *m*-aminophenylboronic acid in one step, as a prelabelling reagent [16]. The reagent has an isoindole fluorophore and a large signal was observed with

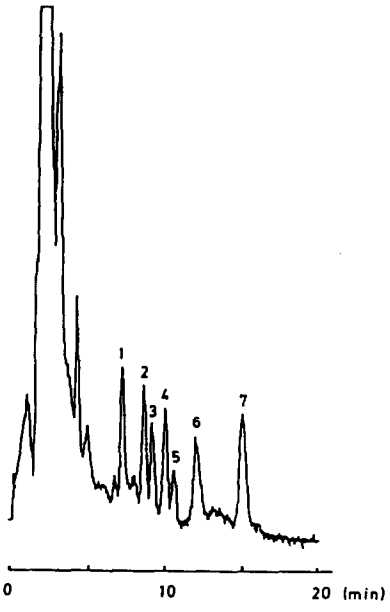

Fig. 3. Chromatogram of phenanthreneboronates of authentic brassinosteroids. Peaks: 1 = 28-norbrassinolide; 2 = brassinolide; 3 = dolichosterone; 4 = 28-norcastasterone; 5 = 28-homobrassinolide; 6 = castasterone; 7 = 28-homocastasterone. Conditions: STR ODS-H column (15 cm × 4.0 mm I.D.); mobile phase, acetonitrile–water (9:1); flow-rate, 0.8 ml/min; temperature, 45°C.

a detector monitoring the fluorescence intensity at 400 nm, when excited at 330 nm. The detection limit for this boronate derivative of brassinolide was 20 pg (signal-to-noise ratio = 3). One of the advantages of this reagent is its high sensitivity in comparison with other boronic acid derivatives used for prelabelling. The separation of five BR boronates, which were formed with the boronic acid in quantitative yield, was successfully carried out with a variety of solvent mixtures under isocratic conditions. The boronates of BRs exhibited sharp peaks, as shown in Fig. 4. The cause of the slightly enhanced broadening of the peak of a CS derivative is not clear.

Third, the prelabelling derivatization of BRs as their dansylaminophenylboronates using fluorimetric detection was studied [17]. The boronates were easily prepared by the reaction of BRs with dansylaminophenylboronic acid, which was newly synthesized as a fluorogenic reagent for BRs.

The excitation maximum, which is representative of the other BR derivatives, is at 345 nm and the emission maximum is at 515 nm. As an example, the detection limit for the boronate derived from BL was 25 pg (signal-to-noise ratio = 2) in the reversed-phase mode. A twofold increase in detectability was observed when the fluorescence of the dansylaminophenylboronates was compared with that of phenanthreneboronates.

The separation of six BRs was successfully performed with a variety of solvent mixtures under isocratic conditions. When a reversed-phase column was used at 45°C with acetonitrile–water (8:2, v/v) as the mobile phase at a flow-rate of 1.0 ml/min, the boronates of BRs exhibited sharp peaks and the derivatives could be separated as shown in Fig. 5.

The relationships between the peak areas and the amounts of the individual BRs were linear from 25 pg to 40 ng. The precision was established by repeated determinations ($n = 8$) using a mixture of BL and CS. The relative standard

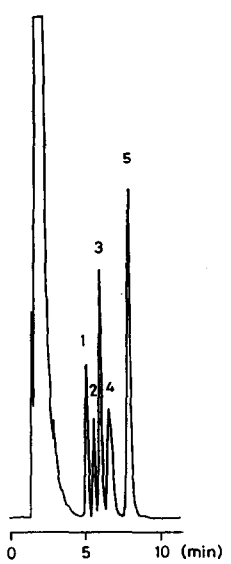

Fig. 4. Chromatogram of 1-cyanoisoindole-2-*m*-phenylboronates of authentic brassinosteroids. Peaks: 1 = brassinolide; 2 = 28-norcastasterone; 3 = 28-homobrassinolide; 4 = castasterone; 5 = 28-homocastasterone. Conditions: STR ODS-H column (15 cm × 4.0 mm I.D.); mobile phase, acetonitrile–1% acetic acid (9:1); flow-rate, 0.8 ml/min; temperature, 45°C.

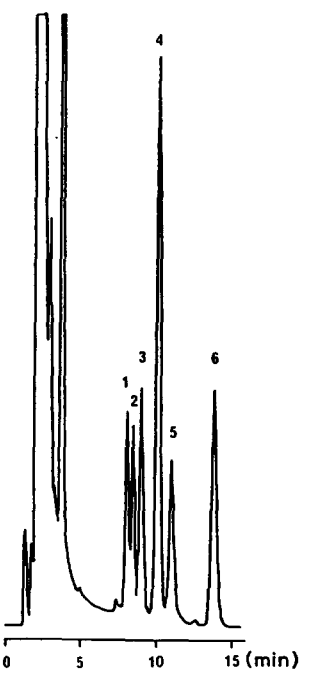

Fig. 5. Chromatogram of dansylaminophenylboronates of authentic brassinosteroids. Peaks: 1 = brassinolide; 2 = dolichosterone; 3 = 28-norcastasterone; 4 = 28-homobrassinolide; 5 = castasterone; 6 = 28-homocastasterone. Conditions: Shim-pack CLC-ODS(M) column (15 cm × 4.6 mm I.D.); mobile phase, acetonitrile–water (8:2); flow-rate, 1.0 ml/min; temperature, 45°C.

deviations (R.S.D.s) were 2.5% and 3.1%, respectively.

As described above, it is evident that the boronic acid derivatives incorporating a fluorophore are suitable and highly sensitive derivatization reagents for BRs that have 1,2-diol groups in the molecules.

6. ELECTROCHEMICAL DETECTION

Electrochemical detection in LC, which was presented by Kissinger *et al.* [18], was applied to the determination in biological samples of catecholamines, catecholestrogens, etc., having a phenolic hydroxyl group or a catechol moiety in the molecules. Further interest in the analysis of BRs prompted us to investigate electrochemical detection in the hope of developing a more sensitive and selective method.

Ferrocene derivatives are readily oxidizable and selectively detected in the presence of other electroactive compounds. Brooks and Cole [19,20] proposed ferroceneboronic acid (FBA) for the prelabelling of glycol compounds for GC with electron-capture detection. We have developed a micro-scale method for the determination of BRs as ferroceneboronate derivatives by HPLC with electrochemical detection [21].

On treatment with FBA, authentic BRs were quantitatively derivatized. The separation of the ferroceneboronates could be performed successfully on a reversed-phase column using acetonitrile–water (85:15, v/v) containing 1 *M* sodium perchlorate as the mobile phase. The hydrodynamic voltammogram of the BL ferroceneboronate derivative showed a constant value above +0.6 V *vs.* a silver–silver chloride reference electrode owing to oxidation of the ferrocenyl moiety. The authentic BR ferroceneboronates were clearly separated on a Shim-pack CLC-ODS(M) column, as illustrated in Fig. 6. The relationships between the peak areas and the amounts of the individual BRs were linear from 50 pg to 5 ng. The method with FBA gave a detection limit for BL of 25 pg per injection (signal-to-noise ratio = 3). A twofold increase in detectability was observed when compared with the fluorimetric detection of phenanthereneboronates. The detection limits of the derivatives with electrochemical detection were found to be comparable to those for other derivatives with fluorimetric detection.

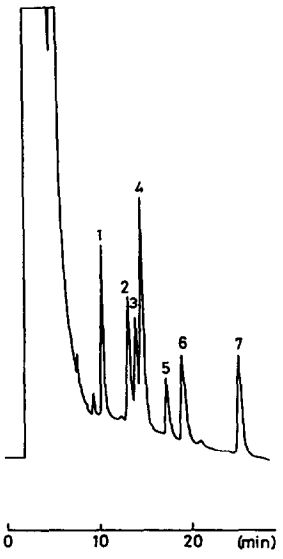

Fig. 6. Chromatogram of ferroceneboronates of authentic brassinosteroids. Peaks: 1 = 28-norbrassinolide; 2-brassinolide; 3 = dolichosterone; 4 = 28-norcastasterone; 5 = 28-homobrassinolide; 6 = castasterone; 7 = homocastasterone. Conditions: Shim-pack CLC-ODS(M) column (15 cm × 4.6 mm I.D.); mobile phase, acetonitrile–water (85:15) containing 1 *M* $NaClO_4$; flow-rate, 1.0 ml/min; temperature, 40°C.

7. SEPARATION OF C-24 EPIMERIC PAIRS OF BRASSINOSTEROIDS

For the microanalysis of BRs, there is an important consideration with respect to the stereochemistry of BR biosynthesis. Much interest has been directed to the stereochemistry at C-24 of BRs in plants, in connection with the stereochemistry of phytosterols. Identification of 24-epiBL in the pollen of broad bean (*Vicia faba* L.) was achieved by means of the selected-ion monitoring technique in GC–MS [22]. We have reported a simple and effective method for the separation of the C-24 epimeric pairs of BL and CS by HPLC with precolumn labelling via the two diol groups and subsequent postcolumn

fluorescence detection [23]. The standard C-24 epimeric mixture of the BRs was derivatized with *m*-aminophenylboronic acid. The boronate formed was injected into a Shim-pack RPC-ODS column. The optimum mobile phase for the separation of the boronates was acetonitrile–1% (v/v) acetic acid (75:25, v/v) containing 20 m*M* 18-crown-6 at a flow-rate of 1.0 ml/min. The BR *m*-aminophenylboronates were monitored by fluorescence detection using a postcolumn reaction system [24], with OPA in the presence of cyanide ion. The first postcolumn reagent is OPA in ethanol/carbonate–borate buffer and the second reagent is potassium cyanide in the same buffer. The boronate OPA derivatives were determined with high response by monitoring the fluorescence intensity at 400 nm, with excitation at 330 nm.

The capacity factors (k'), separation factors (α) and resolution values (R_s) of C-24 epimeric BRs on a Shim-pack RPC-ODS column are listed in Table 2. The chromatogram is shown in Fig. 7. It is evident from the data that a good separation of C-24 epimers of BL and CS was attained. Although no plausible explanation can be given for the elution order of each pair of the epimers (24*S*-isomers were eluted before the 24*R*-isomers), it is likely that an aminophenyl group in the boronate introduced at the side-chain (C-22,23) plays an important role in the conformational discrimination of the C-24 epimeric pairs of BRs in the mobile phase containing a crown ether.

TABLE 2

LC SEPARATION OF THE C-24 EPIMERIC PAIRS OF BRASSINOSTEROIDS AS *m*-AMINOPHENYLBORONATES

For LC conditions, see Fig. 7.

Compound	k'	α	R_s
Brassinolide (24*S*)	2.68	1.07	1.18
24-Epibrassinolide (24*R*)	2.84		
Castasterone (24*S*)	4.31	1.08	1.48
24-Epicastasterone (24*R*)	4.53		

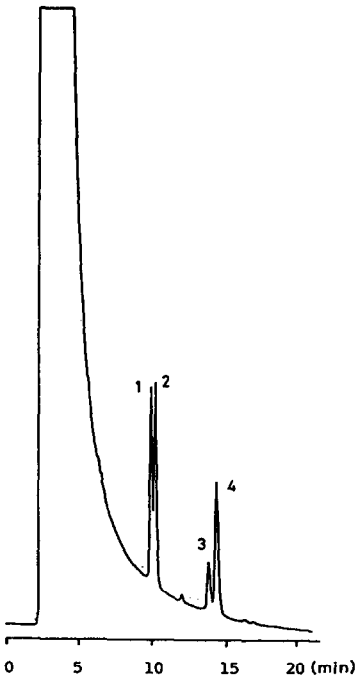

Fig. 7. Chromatogram of the *m*-aminophenylboronate of C-24 epimeric pairs of brassinosteroids. Peaks: 1 = brassinolide; 2 = 24-epibrassinolide; 3 = castasterone; 4 = 24-epicastasterone. Conditions: Shim-pack RPC-ODS column (25 cm × 4.6 mm I.D.); mobile phase, acetonitrile–1% acetic acid (75:25) containing 20 m*M* 18-crown-6; flow-rate, 1.0 ml/min; temperature, 45°C.

8. APPLICATION TO NATURAL BRASSINOSTEROIDS

As it is well known that the content of BRs in plants is very low, the isolation of BRs in a pure form is time consuming and tedious. Therefore, HPLC of trace amounts of BRs contained in partially purified fractions has been investigated. BRs should be converted into suitable derivatives. Our HPLC method was based on esterification of BRs with boronic acid derivatives such as naphthaleneboronic acid [13], 9-phenanthreneboronic acid [15], 1-cyanoisoindole-2-*m*-phenylboronic acid [16], dansylaminophenylboronic acid [17] and ferroceneboronic acid [21]. The derivatized BRs were effectively separated by a reversed-phase column and they were monitored by UV, fluorimetric or electrochemical detection with detection limits of *ca.* 20–100

pg, depending on the prelabelling reagent. The HPLC method is very convenient, highly sensitive and specific. Some examples of its application to the determination of natural BRs are presented below.

When a sufficient amount of BRs was found to be present in a plant material by the rice-lamina inclination test, a crude BR fraction could be used to identify known BRs as boronic acid derivatives. As an example, the identification of BRs in the pollens of broad bean (*Vicia faba* L.) [15] and sunflower (*Helianthus annuus* L.) [25] has been reported. The methanol extract of the respective pollen was subjected to solvent partitioning to obtain a chloroform-soluble neutral fraction. Purification of the fraction by silica gel column chromatography was followed by normal-phase preparative thin-layer chromatography (p-TLC) to give a highly bioactive fraction. The fraction was derivatized with 9-phenanthreneboronic acid and the resulting boronate derivative was subjected to reversed-phase HPLC with fluorimetric detection of (excitation at 305 nm, emission at 375 nm). Four BRs, BL (181 ng/g), dolichosterone (537 ng/g), 28-norCS (628 ng/g) and CS (134 ng/g), were identified in broad bean pollen (Fig. 8) and three

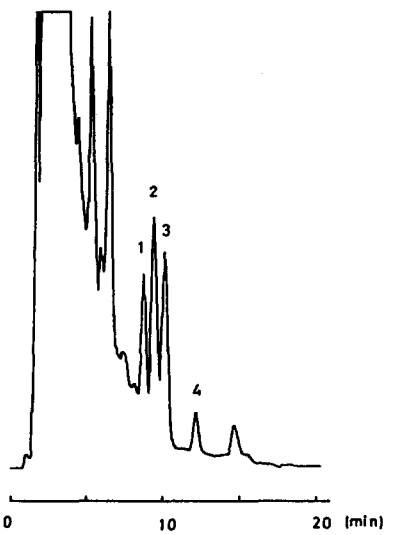

Fig. 8. Chromatogram of the 9-phenanthreneboronates of natural brassinosteroids extracted from the pollen of broad bean. Peaks: 1 = brassinolide; 2 = dolichosterone; 3 = norcastasterone; 4 = castasterone. Conditions as in Fig. 3.

BRs, 28-norCS (65 ng/g), CS (21 ng/g) and BL (106 ng/g), in sunflower pollen.

For comparison of the three fluorescence prelabelling reagents, 9-phenanthreneboronic acid, 1-cyanoisoindole-2-*m*-phenylboronic acid and dansylaminophenylboronic acid, and an electrochemical prelabelling reagent, ferroceneboronic acid, these four reagents were used in analysis for BRs contained in the bioactive fraction obtained from sunflower pollen [9]. Judging from the chromatograms obtained from the derivatized fractions, dansylaminophenylboronic acid is the most effective, because BR derivatives derived from this reagent can be detected at longer wavelengths than those from the other fluorescence reagents. A chromatogram obtained from the derivatives is less susceptible to interference from the matrix than that from the other derivatives, including ferroceneboronate derivatives [9].

As an example of the analysis of a plant material with a low content of BRs, the identification of BRs in buckwheat (*Fagopyrum esculentum* Moench) pollen [26] is described. As the content of BRs in the pollen was found to be very low by bioassay and the BRs were extremely contaminated with unknown natural products, several chromatographic methods, including silica gel adsorption chromatography, normal-phase p-TLC, activated charcoal chromatography and reversed-phase p-TLC, were employed. Aliquots of the bioactive fraction obtained were derivatized with 9-phenanthreneboronic acid and dansylaminophenylboronic acid, respectively. The resulting respective boronate derivatives were subjected to reversed-phase HPLC with fluorimetric detection (excitation at 305 nm, emission at 375 nm and excitation at 345 nm and emission at 515 nm, respectively). As shown in Fig. 9, BL (5.0 ng/g) and CS (7.1 ng/g) were determined in buckwheat pollen.

The results obtained for the three pollens showed a good correlation with those given by GC–MS and BRs identified in the three pollens were also rigorously characterized as bismethaneboronate derivatives by GC–MS [15,25,26]. Our HPLC method using prelabelling reagents is a convenient microanalytical method

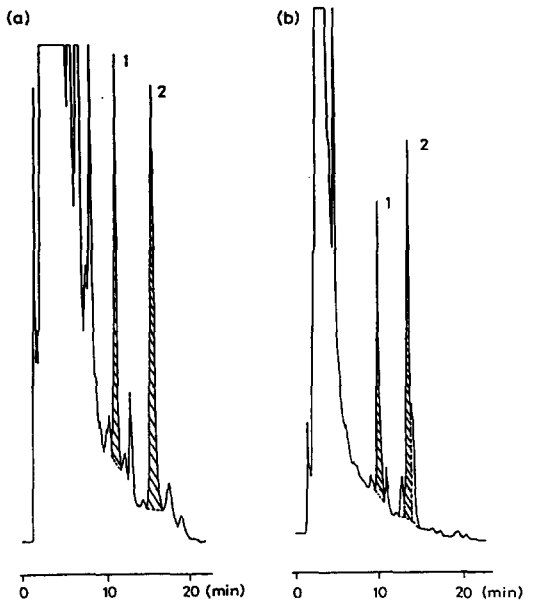

Fig. 9. Chromatograms of (a) 9-phenanthreneboronates and (b) dansylaminophenylboronates of natural brassinosteroids extracted from the pollen of buckwheat. Peaks: 1 = brassinolide; 2 = castasterone. Conditions as in Figs. 3 and 5.

and could be applicable to the identification and determination of trace levels of known BRs in small amounts of plant material because of its high sensitivity and specificity.

9. CONCLUSION

This review has summarized work on HPLC methods for the determination of BRs with UV, fluorimetric and electrochemical detection using boronic acid derivatives as derivatization reagents. We have demonstrated their usefulness in the identification and determination of several BRs in plants. As boronate derivatives were found to be highly sensitive, specific and suitable derivatives, this derivatization method for BRs in HPLC is very useful and ideally suited to this type of sample-limited natural product analysis and may be a suitable tool for use by agricultural and biological chemists interested in natural small amounts of BRs and their trace analysis.

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