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Ferroceneboronic acid as a derivatization reagent for the determination of brassinosteroids by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A micro-scale method for the determination of brassinosteroids as ferroceneboronates by high-performance liquid chromatography with electrochemical detection has been developed. Ferroceneboronic acid (FBA) proved to be satisfactory for use in the derivatization of brassinosteroids with respect to reactivity and sensitivity. The steroids were readily condensed with FBA under mild conditions to provide the corresponding boronates, exhibiting maximum sensitivity at +0.6 V *vs.* a silver–silver chloride reference electrode with a detection limit of 25 pg for brassinolide. The method was successfully applied to the determination of natural brassinosteroids in a plant. Brassinolide, dolichosterone, norcastasterone and castasterone were identified in the pollen of sunflower (*Helianthus annuus* L.).

INTRODUCTION

High-performance liquid chromatography (HPLC) is useful for the separation and determination of trace amounts of various biologically active compounds in many kinds of natural products. Since the discovery of brassinolide as a plant-growth hormonal steroid[1], a number of related steroids, brassinosteroids, have been found to occur in a wide variety of higher plans[2]. Because of their remarkable biological activities and the very small amounts contained in plants[2], micro-scale methods have been necessary for the screening and identification of brassinosteroids in plants. In a previous paper, we described the microdetermination of brassinosteroids using labelling with 1-naphthaleneboronic acid[3], 9-phenanthreneboronic acid[4], 1-cyanoisoindole-2-*m*-phenylboronic acid[5] or dansylaminophenylboronic acid[6]. The boronic acid derivatives were very useful labelling reagents for brassinosteroids which have two

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sets of vicinal diol functional groups, in the A ring and in the side-chain. Further interest in brassinosteroid analysis prompted us to investigate electrochemical detection in hope of developing a more sensitive and selective method.

Shimada and co-workers[7,8] reported that ferrocene reagents, *e.g.*, N-succinimidyl 3-ferrocenylpropionate or ferrocenoyl azide, were very useful for the precolumn labelling of amines or alcoholic hydroxyl compounds for HPLC with electrochemical detection. The ferrocene derivatives are readily oxidizable and selectively detected in the presence of other electroactive compounds. Brooks and Cole[9,10] have already proposed ferroceneboronic acid (FBA) for the precolumn labelling of glycol compounds for gas chromatography with electron-capture detection. However, this reagent does not appear to have been used in HPLC analysis. In this paper, we describe a micro-scale method for the determination of brassinosteroids as ferroceneboronate derivatives by HPLC with electrochemical detection and its application to the identification of brassinosteroids in plant extracts.

EXPERIMENTAL

Chemicals

Authentic brassinosteroids, 28-norbrassinolide, brassinolide, dolichosterone, 28-norcastasterone, 28-homobrassinolide, castasterone and 28-homocastasterone, were synthesized in our laboratory[11–13]. FBA was purchased from Tokyo Kasei (Tokyo, Japan). All other reagents were of analytical-reagent grade.

Plant material and the bioactive brassinosteroid fraction[4]

The bee-collected pollen of sunflower (*Helianthus annuus* L.) was obtained from China and was kindly supplied by Nippon Kayaku (Tokyo, Japan). Identification of the pollen was carried out by microscopic examination. The bioactive brassinosteroid fraction was obtained from the pollen as described previously[14].

Derivatization

The standard mixture or the biologically active brassinosteroid fraction was dissolved in 100 μl of acetonitrile and 100 μl of FBA (1 mg/ml) in 1% (v/v) pyridine–acetonitrile were added. The mixture was heated at 70°C for 10 min. After cooling, several microlitres of the solution were injected directly into the analytical column.

HPLC analysis

A Shimadzu Model LC-6A chromatograph equipped with a Shimadzu Model L-ECD-6A electrochemical detector using a glassy carbon working electrode was employed. A reversed-phase Shim-pack CLC-ODS(M) (5 μm) column (250 mm × 4.6 mm I.D.) was used at 45°C. Samples were injected into the column using a Rheodyne Model 7125 rotary valve syringe-loading injector. The optimum mobile phase for the separation of the brassinosteroid ferroceneboronates was a mixture of acetonitrile and water containing 1 *M* sodium perchlorate at a flow-rate of 1.0 ml/min.

RESULTS AND DISCUSSION

HPLC analysis

On treatment with FBA, the authentic brassinosteroids were quantitatively derivatized in 10 min. In previous studies[3,6], the chromatographic behaviour of brassinosteroids as their boronate derivatives on a reversed-phase column was investigated. Using the data obtained, the separation of brassinosteroid ferroceneboronates could be performed successfully on a reversed-phase column using aqueous acetonitrile containing sodium perchlorate as a mobile phase. When sodium acetate solution was used as the mobile phase, a poor detection limit was observed. As shown in Fig. 1, the hydrodynamic voltammogram of the brassinolide 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 brassinosteroid ferroceneboronates were clearly separated by HPLC on the Shim-pack CLC-ODS(M) reversed-phase column, as illustrated in Fig. 2a. The amounts of these steroids are 10 ng each.

We examined the detection limits of these brassinosteroid derivatives using the ODS column and acetonitrile–water (85:15) containing 1 *M* sodium perchlorate as the mobile phase. The method with FBA gave a detection limit for brassinolide of 25 pg per injection with a signal-to-noise ratio of 3. A two-fold increase in detectability was observed in comparison with the fluorimetric detection of phenanthreneboronates[4]. The detection limits of the derivatives with electrochemical detection were found to be comparable to those of the other derivatives with fluorimetric detection[5,6]. The relationships between the peak areas and the amounts of the individual brassinosteroids were linear from 50 pg to 5 ng. The ferroceneboronates were found to be stable for 2 months in solution in pyridine–acetonitrile at 0°C.

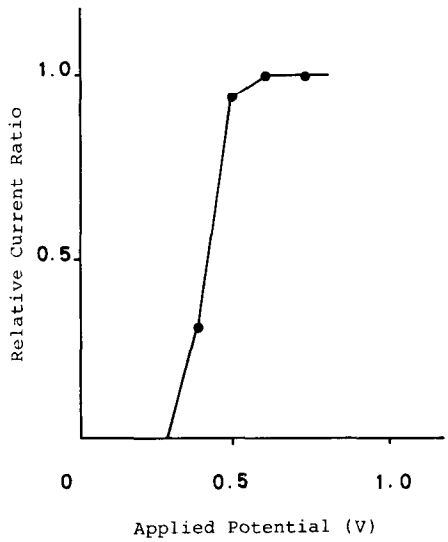

Fig. 1. Hydrodynamic voltammogram of brassinolide ferroceneboronate. The maximum response of the derivative is arbitrarily taken as 1.0.

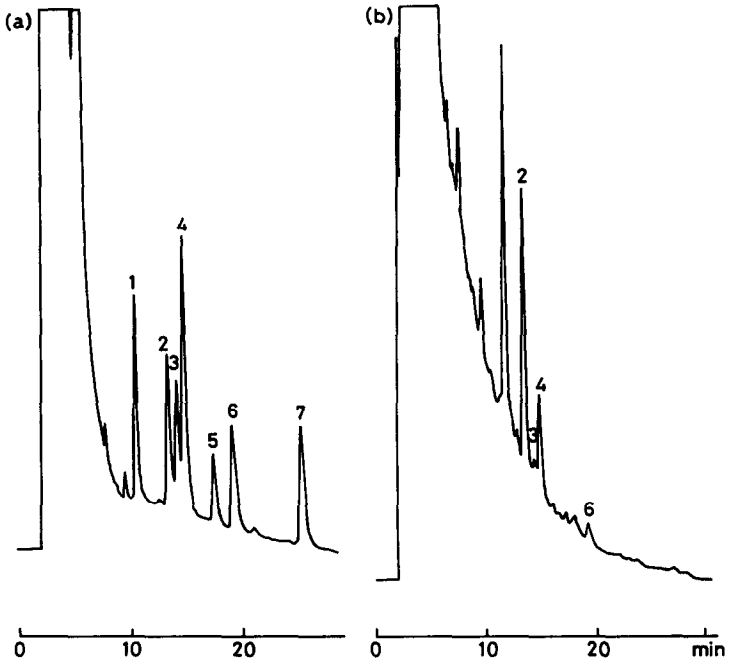

Fig. 2. Chromatograms of brassinosteroid ferroceneboronates. (a) A mixture of authentic samples; (b) biologically active fraction extracted from the pollen of sunflower. Peaks: 1 = norbrassinolide; 2 = brassinolide; 3 = dolichosterone; 4 = norcastasterone; 5 = homobrassinolide; 6 = castasterone; 7 = homocastasterone. Conditions: Shim-pack CLC-ODS(M) column; mobile phase, acetonitrile–water (85:15) containing 1 *M* $NaClO_4$; flow-rate, 1.0 ml/min; temperature, 40°C.

Application

The method was applied to the identification and quantification of brassinosteroids in the pollen of sunflower. The biologically active fraction obtained from the pollen as described previously[14] was derivatized with FBA as described above and analysed by HPLC. As shown in Fig. 2b, brassinolide, dolichosterone, norcastasterone and castasterone were identified in the pollen. The structures of these steroids are shown in Fig. 3. The amounts of these brassinosteroids were determined by using authentic samples for calibration, and the results, except for dolichosterone, were in good agreement with those obtained previously using fluorescence HPLC with 9-phenanthreneboronic acid derivatization and gas chromatography–mass spectrom-

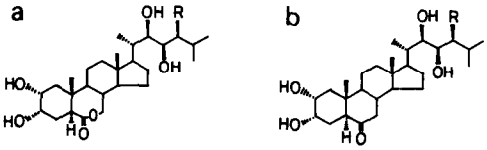

Fig. 3. Structures of brassinosteroids identified in the pollen of sunflower. (a) R = CH_3: brassinolide; (b) R = CH_2: dolichosterone; R = H_2: norcastasterone; R = CH_3: castasterone.

etry[14]. Although dolichosterone was not identified and determined in the same pollen in the previous study[14], the chromatographic data obtained by the present method suggest the presence of dolichosterone in the pollen. Hence it seems that FBA may be a very useful and selective reagent for the derivatization of brassinosteroids.

A recovery test was carried out by adding a mixture of 2 ng of brassinolide and 5 ng of castasterone to the divided bioactive fraction. The samples ere derivatized as described above and analysed by HPLC. The recovery of the added compounds was than >95% ($n = 4$; relative standard deviation = 1.8%). It is evident from these data that the proposed analytical procedure is satisfactory in both accuracy and precision.

In conclusion, this paper is the first report of the application of HPLC with electrochemical detection using FBA as a derivatization reagent for the determination of brassinosteroids. We have demonstrated its usefulness in the identification and quantification of several brassinosteroids in sunflower pollen. As the ferrocene-boronates were found to be highly sensitive, specific and suitable derivatives, this method is ideally suited to sample-limitted natural product analysis and may be suitable for use by agricultural and biological chemists interested in small amounts of natural brassinosteroids and their trace analysis.

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