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Rapid determination of 20-hydroxyecdysteroids in complex mixtures by solid-phase extraction and mass spectrometry

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ABSTRACT

Ecdysteroids possessing a 20,22-diol group react smoothly with arylboronic acids. Cyclic boronates formed in this reaction are stable towards moisture. The changed chromatographic properties of ecdysteroid boronates in comparison with free ecdysteroids allow the efficiency of sample prepurification to be improved using solid-phase extraction. Parent ecdysteroids can easily be released from their boronates by hydrogen peroxide. A rapid and efficient method for the determination of ecdysteroids in samples of biological origin based on solid-phase extraction and fast atom bombardment mass spectrometry is described.

INTRODUCTION

Ecdysteroids are widely distributed natural compounds occurring in both plants and invertebrates. They regulate a series of important physiological functions mostly in insects and other arthropods [1]. However, their function in plants is still not very clear [2,3]. Almost 200 structural analogues have been isolated from plant and ánimal sources so far [4]. They occur in biological material predominantly in complex mixtures. In plants they are often represented as one or two major constituents with admixtures of various minor structurally and biogenetically relative substances. To detect these constituents in either chemical or biological screenings new methods are being developed for their determination or specific extraction [5–7]. For many phytochemical studies there is a need for a rapid method for the determination of ecdysteroids directly in complex mixtures of other extractives. This paper describes such a method, which involves derivatization of ecdysteroids with arylboronic acids followed by solid-phase extraction (SPE) and fast atom bombardment mass spectrometric (FAB-MS) detection of ecdysteroid boronates and/or free ecdysteroids.

Arylboronic acids (aryldihydroxyboranes) have been demonstrated to form stable cyclic boronates with the side-chain diol group of 20-hydroxyecdysteroids (Fig. 1). The versatility of these derivatives has been demonstrated in many instances, *e.g.*, for chromatography [8–10], mass spectrometry [11] and the selective protection of the diol group of ecdysteroids and related compounds [12,13]. Solid-supported phenylboronic acid [6,7] offers the general advantages of solid-supported reagents, *i.e.*, easy removal of non-retained compounds and the possibility of re-using the reagent. On the other hand, it requires an efficient and mild method for releasing retained compounds. A method which is mild with respect to both dihydroxyborane groups and bonded ecdysteroids.

Phenylboronates of ecdysteroids can easily be prepared in various solvents. Several agents, such as diols, hydroxy acids and dicarboxylic acids, have been used for liberating ecdysteroids

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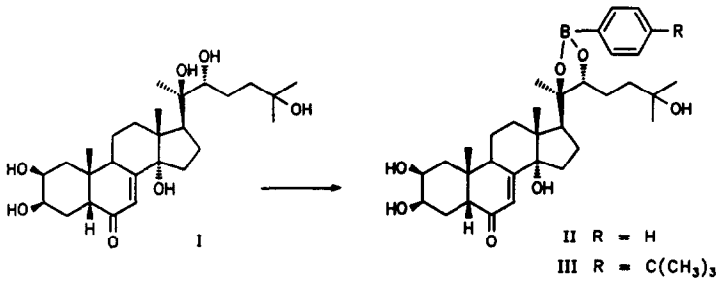

Fig. 1. Structures of 20-hydroxyecdysone (I) and its arylboronates II and III.

from their boronates; however, oxidative splitting of the C–B bond by hydrogen peroxide provides better results [14]. From a practical point of view, it is important that ecdysteroid boronates can also be prepared in water-containing solvents, which are commonly used for the preparation of biological samples.

Changes in the chromatographic properties of boronates permit the use of solid-phase extraction on reversed-phase supports with solvents of higher elutropic strength in comparison with the solid-phase extraction of free ecdysteroids. The efficiency of this purification step is increased when using a solvent with higher elutropic strength to remove polar impurities. In principle, there are two methods to increase the content of ecdysteroids in the sample. One method utilizes the possibility of eluting ecdysteroids as their boronates using a solvent with a higher elutropic strength than is possible when eluting free ecdysteroids. This approach is particularly useful for samples in which the main impurities are eluted with a solvent of the same or slightly higher elutropic strength than for free ecdysteroids. In the other method, the main impurities are eluted with a solvent of lower elutropic strength than is necessary for the elution of ecdysteroid boronates. Subsequently it is possible to release free ecdysteroids from their boronates with a methanolic solution of hydrogen peroxide with an elutropic strength set for the elution of free ecdysteroids. The impurities that would normally be eluted together with free ecdysteroids are thus removed while ecdysteroids are retained as boronates.

Ecdysteroid boronates are formed in complex biological samples in high yields. An excess of arylboronic acid has to be used in order to ensure a quantitative course of the derivatization reaction in water-containing samples and also in samples with a low content of ecdysteroids [14].

Fast atom bombardment mass spectrometry (FAB-MS) has been widely used for the structural identification of ecdysteroids [15–17]. Because it requires only a small amount of sample that, moreover, is applied to a liquid matrix as a solution in protic solvents, FAB-MS appears to be a very efficient method for the determination of ecdysteroids in prepurified samples. Depending on the chosen purification method, ecdysteroids can be determined either as boronates and then released by addition of hydrogen peroxide to the sample dissolved in the matrix on the probe tip, or they can be determined directly in a free form after elution from a solid-phase extraction (SPE) cartridge with a methanolic solution of hydrogen peroxide.

EXPERIMENTAL

Chemicals

Arylboronic acids, *i.e.*, phenylboronic acid and *p*-(*tert.*-butyl)phenylboronic acid, were prepared from the corresponding aryl bromides via Grignard reagents according to a common procedure [18]. 20-Hydroxyecdysone was isolated from roots of *Leuzea carthamoides* (Willd.) DC. The extract from *Leuzea carthamoides* for SPE experiments was prepared from finely ground roots. The roots were extracted with methanol–water (1:1, v/v) four times. The combined extracts were evaporated to half their volume and then extracted with *n*-butanol. The butanolic extract was evaporated to dryness and the residue was dissolved in methanol–water before being subjected to SPE. The content of 20-hy-

droxyecdysone in this extract was *ca.* 10%. Glycerol (Lachema, Brno, Czech Republic) and thioglycerol (Aldrich-Chemie, Steinheim, Germany) were used as the FAB-MS matrix. Hydrogen peroxide (30% aqueous solution) was supplied by Lachema. Methanol (Lachema) was redistilled and water was deionized and distilled.

Derivatization of ecdysteroids using arylboronic acid

20-Hydroxyecdysone 20,22-phenylboronate (**II**) and 20-hydroxyecdysone 20,22-*p*-(*tert.*-butyl)phenylboronate (**III**) were prepared by addition of 1.2 equiv. of arylboronic acid to a solution of 20-hydroxyecdysone (**I**, 10 mg) in methanol (20 μl). The mixture was allowed to react for 5 min. For plant extracts, 5 mg of arylboronic acid were added to the crude extract (20 mg) dissolved in methanol–water mixture (1:1, v/v) (1 ml). The mixture was allowed to react for 10 min.

Solid-phase extraction

SPE of standard compounds **I**, **II** and **III** was performed on Sep-Pak C_{18} cartridges (Waters, Milford, MA, USA). 20-Hydroxyecdysone (**I**) and arylboronates **II** and **III** were applied to Sep-Pak cartridges previously rinsed with water. Methanol–water mixtures of different elutropic strength (2.5 ml) were used for elution. The amounts of eluted compounds **I–III** were determined by weighing of fractions (Fig. 2).

For *Leuzea carthamoides* extract a different method had to be used because of problems with the solubility of crude extracts after the derivatization reaction. A reversed-phase sorbent (40-μm Separon SGX C_{18}; Laboratorní přístroje, Prague, Czech Republic) (40 mg) was added to the solution of derivatized extract and the mixture was evaporated to dryness. The residue was added to a reusable Supelclean SPE tube (Supelco, Bellefonte, PA, USA) filled with the same sorbent (40 mg). The cartridge was enclosed and soaked with water. Methanol–water mixtures (2.5 ml) (containing 60%, 80% and 100% of methanol) were then used for elution.

The ecdysteroid content in the fractions eluted from the SPE cartridge was determined by reversed-phase HPLC. Ecdysteroid boronates

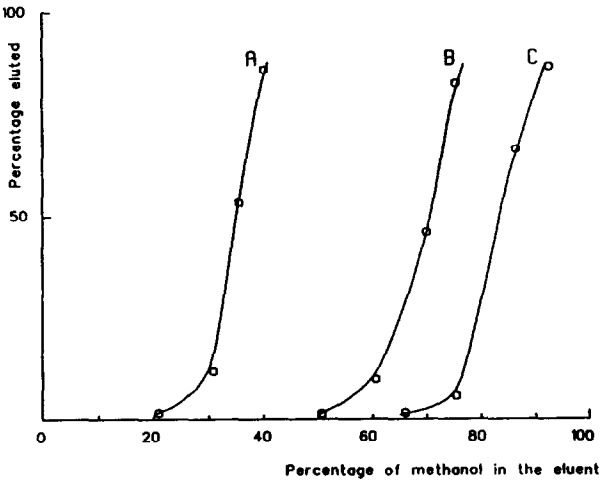

Fig. 2. Elution of (A) 20-hydroxyecdysone (**I**), (B) its phenylboronate (**II**) and (C) *p*-(*tert.*-butyl)phenylboronate (**III**) previously adsorbed on a Sep-Pak C_{18} cartridge. Percentage of 20-hydroxyecdysone (**I**) and its boronates **II** and **III** eluted with 2.5 ml of various methanol–water mixtures.

were first converted into free ecdysteroids by addition of hydrogen peroxide and then the ecdysteroid content in the fractions was determined by reversed-phase HPLC (Table I). A methanolic solution of 20-hydroxyecdysone was used as an external standard.

Mass spectrometry

Mass spectrometric measurements were peformed on a ZAB-EQ reversed-geometry mass spectrometer (VG Analytical, Manchester, UK) with an M-scan FAB gun (xenon, 8 kV, 1 mA) at an accelerating voltage of 8 kV. Glycerol–thioglycerol (3:1, v/v) was used as the matrix. Methanolic fractions from SPE (1 μl) were added to the matrix and mass spectrum was recorded. Release of free ecdysteroids was then accomplished by addition of hydrogen peroxide (3 μl) to a sample dissolved in the matrix on the probe tip. After reaction for 10 min the probe was inserted into the ion source and the spectrum of free ecdysteroid was recorded (Figs. 3 and 4).

RESULTS AND DISCUSSION

The elution curves of 20-hydroxyecdysone (**I**) and its phenylboronate (**II**) and *p*-(*tert.*-butyl)-

TABLE I

RESULTS OF SOLID-PHASE EXTRACTION OF *LEUZEA CARTHAMOIDES* EXTRACT AFTER DERIVATIZATION WITH ARYLBORONIC ACIDS

Content of methanol in eluent (%)	PBA[a]		*t*-BPBA[b]	
	Mass proportion (%)[c]	Content of 20E (%)[d]	Mass proportion (%)[c]	Content of 20E (%)[d]
60	83.4	0.5	67.1	0.3
80	10.2	25.5	23.9	3.0
100	6.4	40.1	9.0	48.7

[a] Extract treated with phenylboronic acid.
[b] Extract treated with *p*-(*tert.*-butyl)phenylboronic acid.
[c] Mass proportion of SPE fractions eluted by the eluent with corresponding elutropic strength.
[d] Content of 20-hydroxyecdysone determined after splitting of boronates.

phenylboronate (**III**) on Sep-Pak C_{18} are shown in Fig. 2. Both boronate curves are shifted to the area of higher methanol content. The presence of bulky *tert.*-butyl group in boronate **III** resulted in even stronger retention in comparison with boronate **II**. As *p*-alkylphenylboronic acids are accessible from related bromides, such aliphatic chains containing arylboronic acids represent an efficient route to modifying the polarity of ecdysteroid boronates. Consequently, the chromatographic properties of boronates can be suitably set for any particular sample. Boronates **II** and **III** showed sufficient stability during the SPE process. When crude extracts of *Leuzea carthamoides* were used, the observed elution curves did not exactly match those of pure boronates **II** and **III**. The major proportion of waste substances (more than 90%) was eluted with the eluent containing up to 80% of methanol. The highest concentration of 20-hydroxyecdysone, *ca.* 40% of the mass of the fraction, was detected (after splitting boronates) in the methanolic fractions. The results are summarized in Table I.

The methanolic fractions were used directly for FAB-MS. The mass spectra of fractions containing boronates **II** and **III** are shown on Figs. 3A and 4A, respectively. The protonated molecular ions of boronates, *m/z* 567 (Fig. 3A)

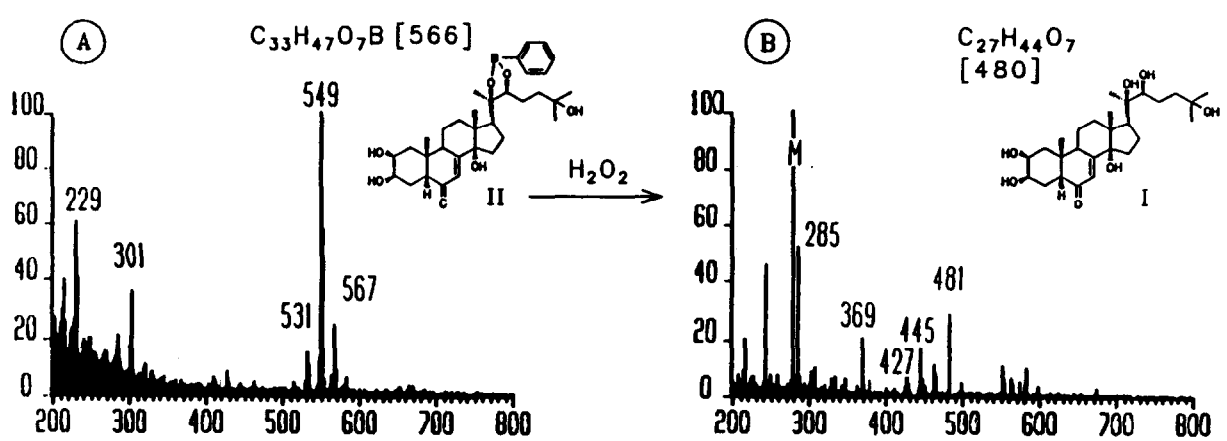

Fig. 3. (A) FAB mass spectra of fraction of *Leuzea carthamoides* extract containing boronate **II**; (B) after the reaction with hydrogen peroxide.

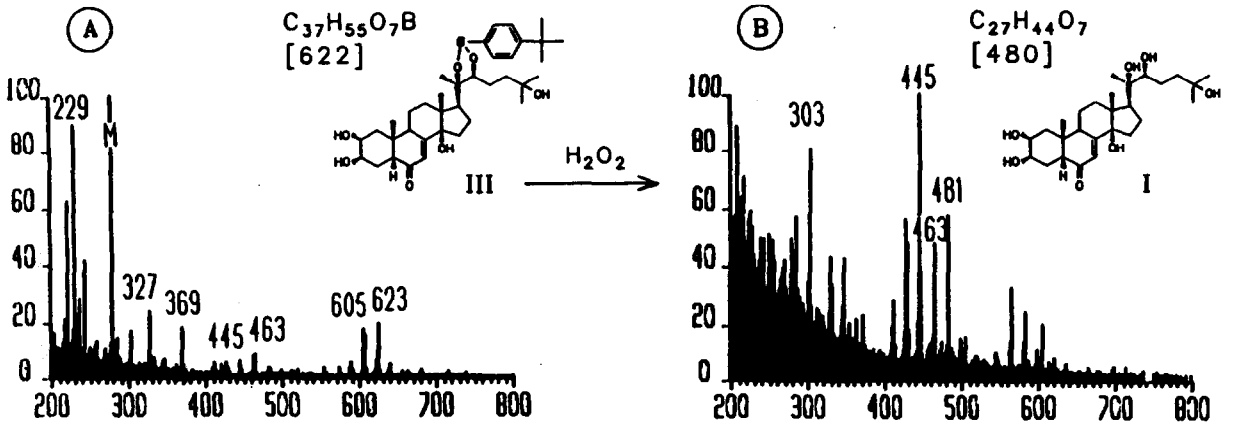

Fig. 4. (A) FAB mass spectra of fraction of *Leuzea carthamoides* extract containing boronate III; (B) after the reaction with hydrogen peroxide.

for derivatization with phenylboronic acid and *m/z* 623 (Fig. 4A) for derivatization with *p*-(*tert.*-butyl)phenylboronic acid, were sufficiently abundant to be easily detected. The spectra obtained after releasing 20-hydroxyecdysone (I) are shown in Figs. 3B and 4B, respectively. The protonated molecular ion of 20-hydroxyecdysone appeared at *m/z* 481 in both instances (Figs. 3B and 4B). By-products of the cleavage of boronates II and III, *i.e.*, boronic acid and phenol or *p*-(*tert.*-butyl)phenol, do not interfere in the range of interest for MS measurements. The ecdysteroid content in both samples was sufficiently high to obtain mass spectra of good quality. As cyclic boronate formation is typical of 20,22-diol-containing ecdysteroids, this reaction and also the reverse splitting can be considered as further evidence of the presence of ecdysteroids. The molecular mass of the present ecdysteroid can be determined from mass spectrum. Further information can be obtained from the fragmentation pattern, but this is often limited to losses of water molecules.

Leuzea carthamoides is known to contain, in addition to 20-hydroxyecdysone, several further minor ecdysteroids also possessing a 20,22-diol group [19]. It may be expected that they are also present in the analysed fractions. However, they were not found in the mass spectra of these complex mixtures, probably because of their low abundance. Experiments with MS measurements under various conditions is required for the determination of these trace ecdysteroids. In natural sources of ecdysteroids there could also be present ecdysone-related compounds not containing a 20-hydroxyl group and/or with a vicinal diol group in the side-chain protected as an isopropylidene or ester derivative. As the 2,3-diol moiety is not able to form stable boronates [14], these compounds could not be determined by this method. The method is limited to the major group of 20-hydroxyecdysteroids.

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