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Boronic esters as derivatives for supercritical fluid chromatography of ecdysteroids

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ABSTRACT

The use of boronic ester derivatives for the recognition of ecdysteroids possessing a 20,22-diol group in supercritical fluid chromatography is demonstrated for some model systems and for insect and plant extracts. Derivative formation with methyl-, butyl- and phenyl-boronic acids occurs rapidly under mild conditions with excess boronic acid to give products of significantly shorter retention than the parent ecdysteroids with supercritical CO_2–methanol mobile phase.

INTRODUCTION

The importance of ecdysteroids (insect moulting hormones) in insect development, and their identification and quantification is a matter of great interest to insect physiologists and biochemists looking for new ways to control insect pests.

Ecdysteroids are found in insect tissues and eggs in μg/g to pg/g quantities [1]. Such small amounts of ecdysteroids require either selective methods of extraction providing sufficient material with high purity for analysis or a method of analysis with sensitive and selective detection [2]. Some of these same compounds and others, known as phytoecdysteroids are found in plants scattered throughout the plant kingdom [3]. The development of sensitive and specific analytical methods for the ecdysteroids, was of fundamental importance in the advance of ecdysteroid research [4].

The ecdysteroids are a family of polyhydroxylated sterols. They are interesting for chromatographic studies because they all have the same rigid 5β-cholestane skeleton, with a 7-ene-6-one UV chromophore and a variety of other substituent groups. We have already demonstrated the advantages of supercritical fluid chromatography (SFC) with CO_2–methanol for the analysis of ecdysteroids [5,6].

Boronic acids can react selectively with 1,2- and 1,3-diols, enediols, 2-hydroxy acids and other such groups to form cyclic derivatives. Brooks and co-workers [7,8] have thoroughly explored the use of boronic acids, including phenylboronic acid to give volatile cyclic boronate derivatives of steroids for gas chromatography. Poole and Morgan [9] explored the use of cyclic phenylboronates as gas chromatographic derivatives for ecdysteroids coupled with the use of the nitrogen-specific detector for selective detection of the boron. Poole *et al.* [10] also suggested phenanthreneboronic acid as a fluorescence label in the HPLC and TLC analysis of ecdysteroids. More recently, we have used phenylboronic acid immobilized on silica gel for the solid-phase extraction of ecdysteroids

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[11,12]. Phenylboronic acid has also been used as derivatization agent in HPLC and TLC [13,14]. These boronic esters have selective mass spectral fragmentations that are helpful in structure identification [13]. The appearance of a chromatogram before and after treatment with a boronic acid spotlights the presence of ecdysteroids with the side-chain diol group.

Because of the constant demand to determine sensitive and specific analytical methods for the ecdysteroids, we have investigated here a number of boronate esters which are formed exclusively from the diol in the side chain of ecdysteroids (Fig. 1), even if an excess of reagent is present in the reaction mixture. The reaction is quantitative and ecdysteroid boronates are stable in supercritical carbon dioxide–methanol mobile phase. The derivatization reaction results in a decrease in polarity of the ecdysteroids. This property can be utilized for simple checking of the presence of the 20,22-diol system in the ecdysteroid molecule and might be extended to SFC–electron-capture detection.

EXPERIMENTAL

Ecdysteroids are obtained from the collection of I.D. Wilson or were purchased from Simes (Milan, Italy). The samples of plant extracts of *Silene otites* and *Ajuga* (tentatively identified as *A. iva*) were gifts of R. Lafont. Ecdysteroids and boronic acids were prepared as dilute solutions in methanol. Varying amounts of methyl, butyl or phenylboronic acid were added to a methanol solution of an ecdysteroid and the reaction mixture was left for 3 min at room temperature before chromatography.

SFC experiments were performed on a purpose-built system (LDC Analytical, Stone, UK) containing guard and analytical columns (LDC Analytical, 150 × 4.6 mm I.D. packed with 5 μm cyanopropyl silica) with carbon dioxide–methanol (9:1) as mobile phase at a flow-rate of 1.8 ml/min and a pressure of 320 bar and temperature of 55°C.

Samples (10 μl) were injected into the system through a Rheodyne 7125 injection valve with a 20-μl injection loop (Anachem, Leek, UK). UV detection was set to monitor at 240 nm.

RESULTS AND DISCUSSION

The formation of boronic esters was explored with four representative ecdysteroids. 20-Hydroxyecdysone (**I**) (for structures, see Fig. 1), 20-hydroxyecdysone-2-cinnamate (**II**) and cyasterone (**III**) all possess 20,22-diols and all formed cyclic boronates. Ecdysone (**IV**) which possesses a 2,3-diol but not a 20,22-diol showed no evidence of formation of a cyclic ester under mild conditions even with a large excess of reagent. This is consistent with our earlier finding in a study of the use of immobilized boronic acids for solid-phase extraction of ecdysteroids [11] (see also refs. 13 and 14). Only those ecdysteroids with a 20,22-diol were selectively retained on the boronic acid column through cyclic ester formation [11,12]. The distance between the hydroxyl groups on C-20 and C-22 is suitable for formation of the cyclic boronate

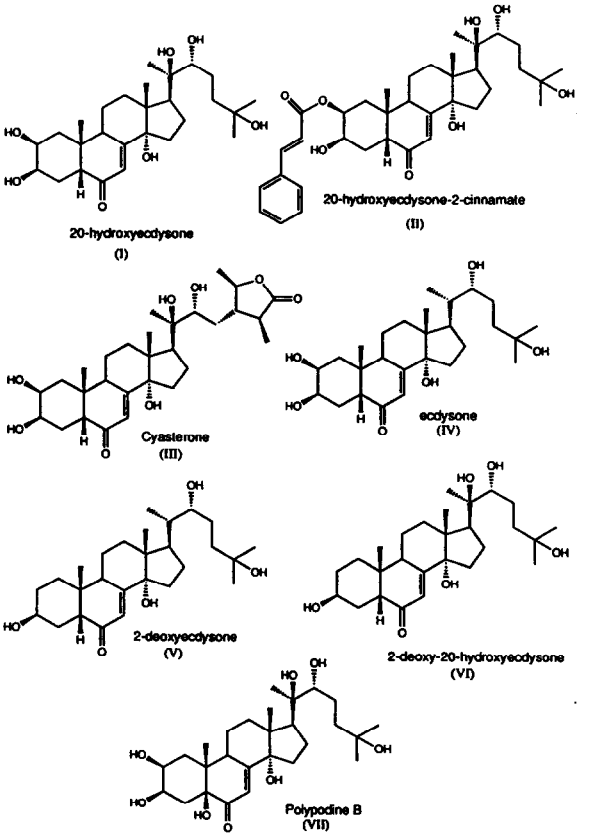

Fig. 1. Structures of ecdysteroids studied here.

ester, however *cis* fusion of the A and B rings of ecdysteroids causes the C-2 and C-3 β-hydroxyl groups to be too far apart for easy formation of a cyclic boronate, so, for example, ecdysone was not absorbed onto the immobilized boronic acid column [11]. The same effect appears to be operating here. Ecdysteroid 2,3-*cis*-diols do not give cyclic boronates under the conditions explored.

Addition of an excess of 1% solution of methyl-, butyl- or phenyl-boronic acid to a dilute (micromolar) solution of an ecdysteroid possessing a 20,22-diol gave rapid formation of a stable cyclic boronate ester (Fig. 2) which had a shorter retention time than the parent ecdysteroid. The excess of boronic acid required to convert a known quantity of ecdysteroid to its cyclic ester in 3 min at room temperature was explored with the three boronic acids and 20-hydroxyecdysone. Plots of extent of conversion to boronic ester against quantity of boronic acid added are given in Fig. 3. The formation of the phenylboronic ester occurred much more readily than either the methyl or butyl esters (Fig. 4), which required a ten-fold greater excess of reagent for complete conversion. The electron-donating effect of the phenyl ring evidently assisted ester formation.

Raising the temperature of reaction had little effect on the rate of reaction, but leaving the mixture together for a longer time at room temperature before chromatography gave more complete reaction with amounts of boronic acid below the optimum (Table I).

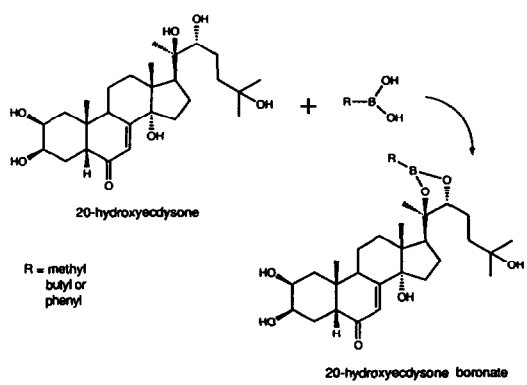

Fig. 2. Formation of a cyclic boronic ester of 20-hydroxyecdysone.

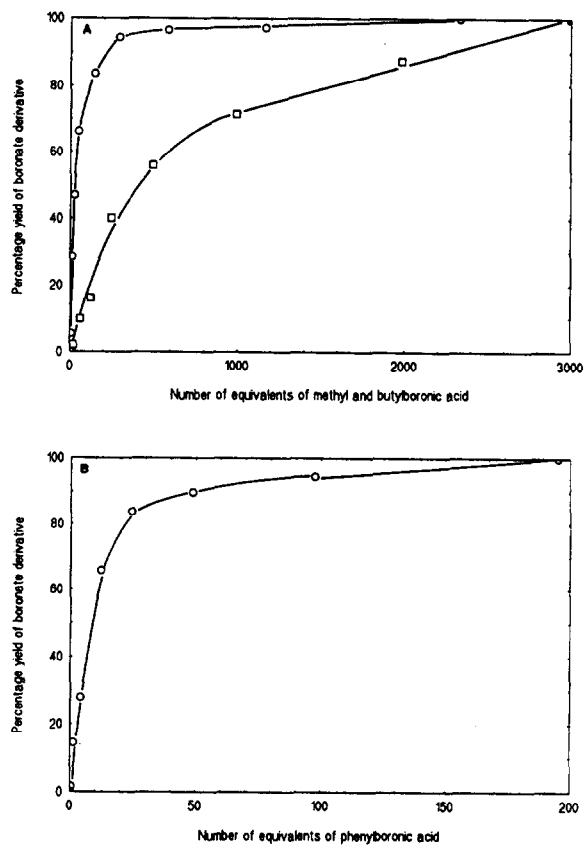

Fig. 3. Plots of extent of reaction of 20-hydroxyecdysone with differing amounts of (A) methyl- and butyl- and (B) phenylboronic acids to show the amount required for complete conversion in 3 min at room temperature. (A) □ = Methylboronate; ○ = butylboronate.

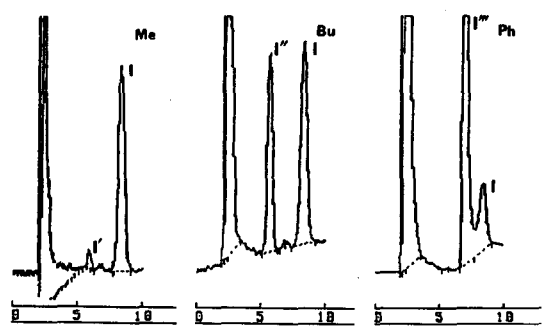

Fig. 4. SFC chromatograms of 20-hydroxyecdysone (I) after reaction with 10 molar excess of methyl-, butyl- and phenylboronic acids for 3 min at room temperature. I′ = Methylboronate; I″ = butylboronate; I‴ = phenylboronate. Time scale in min.

TABLE I

EFFECT OF TIME AND TEMPERATURE ON EXTENT OF REACTION BETWEEN 20-HYDROXYECDYSONE ($4.2 \cdot 10^{-10}$ mol) AND METHYLBORONIC ACID ($4.17 \cdot 10^{-9}$ mol) OR PHENYLBORONIC ACID ($4.0 \cdot 10^{-9}$ mol) IN DILUTE SOLUTION IN METHANOL

Boronic acid	Molar excess	Temperature (°C)	Time (min)	Conversion (%)
Methyl	993	Room	3	67
		Room	5	75
		Room	12	87
		50	3	78
Phenyl	9.5	Room	3	71
		Room	5	78
		Room	30	100
		50	3	74

The three ecdysteroids of varying structure were chosen to observe the change in retention on conversion to the boronic esters. Their relative retentions are shown in Table II. Conversion to a particular boronic ester had very similar effects upon each of the ecdysteroids. For example, the methylboronic esters of all three ecdysteroids (I to III) had retention times 24% shorter than the corresponding free ecdysteroids in the chromatographic system chosen. The butyl esters had shortest retentions and the phenyl esters were the closest to the parent ecdysteroids (Fig. 4).

To test the use of these boronic esters with natural materials, we have examined crude extracts of desert locust (*Schistocerca gregaria*) eggs, which, after enzymic hydrolysis, contain ecdysone (**IV**) and 2-deoxyecdysone (**V**), and extracts of *Ajuga iva* (tentative identification) which contains 20-hydroxyecdysone (**I**), 2-deoxy-20-hydroxyecdysone (**VI**) and polypodine B (**VII**) and an extract of *Silene otites* which we have already examined by SFC [6] and which contains polypodine B (**VII**), and 20-hydroxyecdysone (**I**). The *Schistocerca* eggs contain ecdysteroids which do not form boronic esters, however, although the main peaks were unaffected on addition of boronic acid, a small new peak appeared at 5.5 min, which suggested there was a small amount of complex-forming ecdysteroid in the mixture (Fig. 5A). In the case of the *Ajuga* and *Silene* plant extracts, all the components were shifted to shorter retention times when sufficient boronic acid was added (Fig. 5B, C) showing that all the components in the extract contained the 20,22-diol structure, although compounds **VI** and **VII** eluted as one unresolved peak, as did their boronic esters, under the conditions used (Fig. 5B).

These experiments demonstrate that boronic esters can be formed in neutral media without catalysis and with impure mixtures. These less polar esters can give a rapid indication of 20,22-diol ecdysteroids in crude plant or animal ex-

TABLE II

RELATIVE RETENTION TIMES OF ECDYSTEROIDS AND THEIR CYCLIC BORONATE ESTERS ON A CYANOPROPYL SILICA COLUMN USING SUPERCRITICAL CARBON DIOXIDE–METHANOL (9:1) AS MOBILE PHASE

Ecdysteroid (Fig. 1)	Boronic acid	t_r ecdysteroid[a]	t_r ester	t_r ester/ t_r ecdysteroid
I	Methyl	7.45	5.66	0.76
	Butyl	7.63	5.52	0.72
	Phenyl	7.65	6.48	0.85
II	Methyl	6.51	4.97	0.76
	Butyl	6.51	4.88	0.75
	Phenyl	6.77	5.86	0.87
III	Methyl	8.75	6.64	0.76
	Butyl	8.83	6.50	0.74
	Phenyl	8.87	7.84	0.88

[a] Retention times vary slightly with time, therefore relative retentions are quoted.

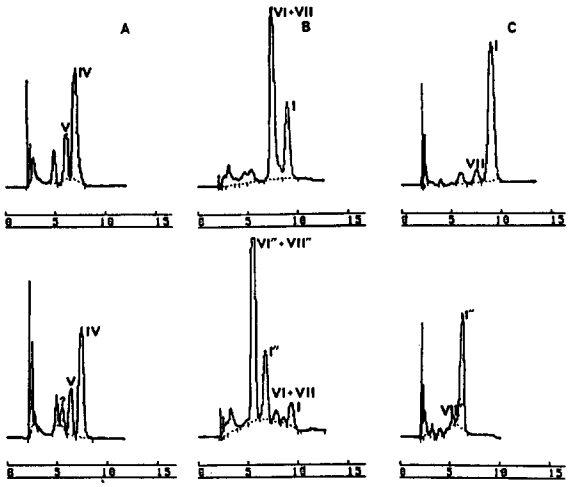

Fig. 5. SFC chromatograms of (A) *Schistocerca* eggs, (B) *Ajuga* (*iva*?) extract and (C) *Silene otites* plant extracts, before (above) and after treatment with butylboronic acid (below). (B) shows incomplete conversion, when using a larger excess of butylboronic acid the residual peaks at 8 and 10 min disappeared. Roman numerals correspond to those in Fig. 1. Roman numerals with double prime indicate the corresponding butylboronate esters. Time scale in min.

tracts and can be a useful guide in the screening of plant materials. For rapid formation, the phenylboronic esters is preferred, but if a larger shift in retention is required, the butylboronic ester is recommended.

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