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Short communication

Reversed-phase affinity chromatography of ecdysteroids with boronic acid-containing eluents

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Abstract

Selective effects of boronic acid-containing eluents on the chromatographic behaviour of ecdysteroids are described. The use of such eluents provides a simple, rapid and sensitive means to assess the presence of certain diol groups in ecdysteroids by ordinary RP-HPLC. It is demonstrated that ecdysteroids with different sets of diols in their molecules have individual shapes of chromatographic peaks. A mechanism of the boronic acid effect is discussed.

Keywords: Mobile phase composition; Ecdysteroids; Boronic acid

1. Introduction

Many ecdysteroids have been described as natural compounds in plants and animals [1,2], and when present they usually occur as complex mixtures of compounds with related structures. Their identification usually relies on the comparison of their chromatographic properties with those of available reference compounds. The final assessment of their structures requires NMR and mass spectrometric analysis of pure substances [1–3].

Chromatography is used for both analytical and preparative purposes, but it is hardly informative when no reference substances are available, especially when new ecdysteroids are concerned. Owing to its special selectivity, affinity chromatography seems to represent a sensitive tool which allows one to assess the presence of a given structure in a molecule. In this respect, the well known ability of boronic acid (and its derivatives) to bind specifically with *cis* vicinal diol groups [4] is of special interest, although the use of immobilized boronic acid on a solid phase has been limited up to the present. The reasons for this limitation may be that the best sorbents with attached phenylboronic groups are polymeric, which restricts their use in HPLC, and most mineral HPLC sorbents are not resistant to the alkaline eluents which are required for the complete realization of boronic acid affinity.

In practice, the present affinity methods of separation concerning ecdysteroids involve the use of solid-phase extraction procedures. They allow one to separate groups of substances on the basis of the presence or absence of a 20,22-diol group [5–8]: ecdysteroids bearing a diol in

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this position are bound to the affinity sorbent under alkaline conditions and eluted after acidification of the mobile phase. Various organic derivatives of boronic acid (e.g., phenylboronic acid) are usually used to prepare relatively stable derivatives of ecdysteroids as either modifiers in chromatography or as protective agents in chemical syntheses [8–13].

An alternative strategy could be the use of boronic acid (or a derivative) as a component of the mobile phase rather than as the modifier of a sorbent, and to our knowledge this has not yet been investigated in HPLC. This paper describes a preliminary approach of the use of boronic acid as a component of the mobile phase in the reversed-phase HPLC of ecdysteroids.

2. Experimental

Water, methanol and butanol were distilled in glass. Boronic acid was of chemically pure grade and sodium chloride and mannitol were of analytical-reagent grade (Reakhim). 20-Hydroxyecdysone, inokosterone and ecdysone (purity >98%) were purchased from Northern Biochemical (Syktyvkar, Russia) and abutasterone, integristerone A and 5,20,26-trihydroxyecdysone were a gift from Professor R. Lafont (CNRS, Paris, France).

The HPLC equipment (pump and UV–Vis detector set at 254 nm) was obtained from Laboratórní Přístroje (Prague, Czech Republic). HPLC columns A and B (150×4 mm I.D.) (particle size 6 and 8 μm, respectively) and column C (250×4 mm I.D.) (particle size 7 μm) with precolumns (10×4 mm I.D.) (particle size 10 μm) were packed with Diasorb-C16/T (Bio-ChemMack, Russia). The flow-rate was 0.7 or 1.0 ml/min and the temperature was 25°C.

The classical RP-HPLC system was modified by adding boronic acid to give 0.005–0.01 *M* solutions in methanol–water (9:11, v/v) eluent and 0.01–0.3 *M* solutions in butanol–water (1:24, v/v) eluent. Boronic acid-free solvents were used for comparison. The exact conditions are given later in the text and in the figure captions.

3. Results and discussion

The basic idea is to use the affinity of boronic acid towards vicinal diols of appropriate configuration. Boronic acid forms either uncharged adducts (**I**) or charged complexes (**II**) with the 20,22-diol group of ecdysteroids (Fig. 1), but it does not react (or to a negligible extent) with the 2,3-diol group [5–7]. There is no information in the literature concerning diols in other positions. The ecdysteroid–boronic acid adducts **I** are less stable in aqueous media than complexes **II**, the formation of which depends strongly on pH. Both product yields also depend on the content and type of organic solvent in aqueous media [7,14]. More complete formation of **II** would require working in the pH range 8–11, but it can be expected that even partial interactions between ecdysteroids and boronic acid would result in some modification of their chromatographic behaviour.

The addition of boronic acid to a methanolic mobile phase results in broadening of the chromatographic peaks corresponding to the ecdysteroids with any vicinal diol system, except 2,3-diol (Figs. 2–4). Under the same conditions, no sensitive changes for ecdysone, with only a 2,3-diol in its molecule, were found (see Fig. 6). The peak broadening is accompanied by an increase in the retention time. It is important to note that

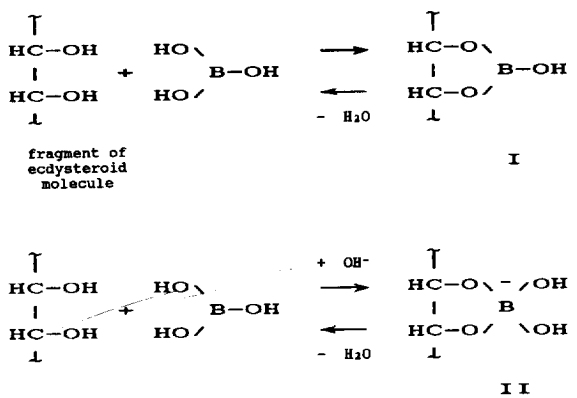

Fig. 1. Scheme of boronic acid reactions with the *cis* vicinal diol group of ecdysteroid molecules.

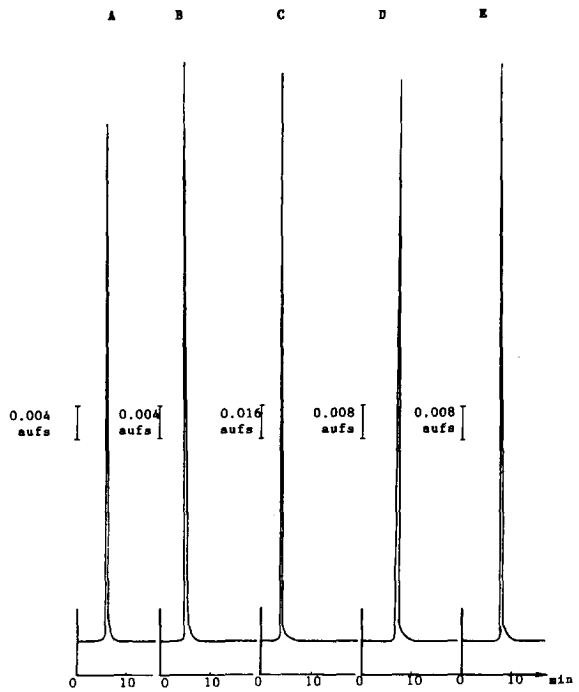

Fig. 2. Chromatograms of individual ecdysteroids. Peaks: A = abutasterone; B = integristerone A; C = 5,20,26-trihydroxyecdysone; D = 20-hydroxyecdysone; E = inokosterone. Column A; eluent, methanol–water (9:11, v/v); flow-rate, 0.7 ml/min.

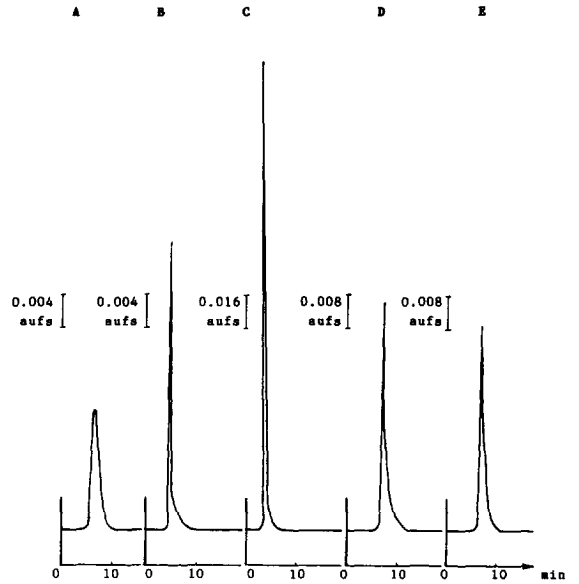

Fig. 3. Chromatograms of individual ecdysteroids. Peaks as in Fig. 2. Column A; eluent, 0.005 *M* boronic acid in methanol–water (9:11, v/v); flow-rate, 0.7 ml/min.

a similar phenomenon, but in RP-TLC, has been described earlier [7]: when added to the eluent, phenylboronic acid led to spreading of the spots for substances with a 20,22-diol function.

It appears that the effect of the addition of boronic acid on an ecdysteroid peak depends both on the presence of a reactable diol and on its position. The peak of abutasterone, the molecule of which contains two reactable diols in 20,22- and 24,25-positions and an "inert" 2,3-group, undergoes the largest broadening (Fig. 3A). The peak of integristerone A (1,2,3-triol and 20,22-diol) shows less broadening (Fig. 3B), while the peak of 5,20,26-trihydroxyecdysone undergoes the smallest change (Fig. 3C). The last result is surprising in comparison with those for 20-hydroxyecdysone and inokosterone; it is as if an additional 25,26-diol inhibits the sensitivity of the 20,22-group to boronic acid (compare Figs. 3C, D and E). It is probable that boronic acid allows the detection both of the presence of

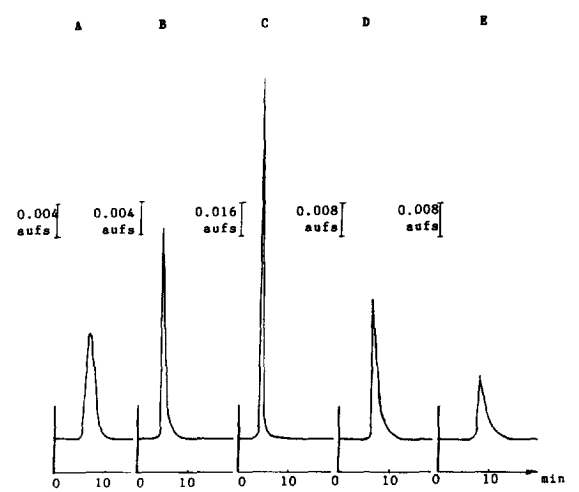

Fig. 4. Chromatograms of individual ecdysteroids. Peaks as in Fig. 2. Column A; eluent, 0.01 *M* boronic acid in methanol–water (9:11, v/v); flow-rate, 0.7 ml/min.

reactable diols and their combination in a molecule, as demonstrated above. Testing a greater number of ecdysteroids with different isolated diols and combinations of diols is required to ascertain more details.

An increase in boronic acid concentration results in a longer retention time and in peak broadening of ecdysteroids possessing a 20,22-diol group or a combination of the latter with 1,2-, 24,25- and 25,26-diol groups (Fig. 4). Boronic acid in butanolic eluents [butanol–water (1:24, v/v)] shows similar effects to those described above for methanolic phases (Fig. 5). This effect of boronic acid is not mimicked by adding either a salt (0.02 *M* NaCl) or an undissociative solute (0.02 *M* mannitol).

The phenomenon under investigation may have roots in different parts of the chromatographic system. It is probable that peak broadening could be a result of an equilibrium formation of some ecdysteroid–boronic acid adducts of type **I**, owing to a local gradient of boronic acid concentration in the chromatographic band of ecdysteroids along the column. In order to test this hypothesis, two samples were analysed, one of them having reacted with boronic acid prior to injection. The results were identical (data are not shown). In addition, tenfold dilution of a sample (i.e., an increasing excess of boronic acid and consequently directing the equilibrium towards adduct formation) only resulted in a proportional decrease in the signals with other chromatographic parameters constant. This means that the reaction has sufficient time for completion during passage of the ecdysteroids through the column, even if the sample does not contain boronic acid.

Nevertheless, changes in the concentration of boronic acid alter the chromatographic behaviour of ecdysteroids bearing a reactable vicinal diol function. It appears impossible to explain the observed phenomenon by equilibrium in the mobile phase. Indeed, if in the liquid phase a compound of type **I** or **II** is formed even in small amounts, it typically possesses individual chromatographic properties (e.g., retention time). This results in the appearance of a new peak and in a proportional decrease in the peak of a parent substance. Such a phenomenon has been

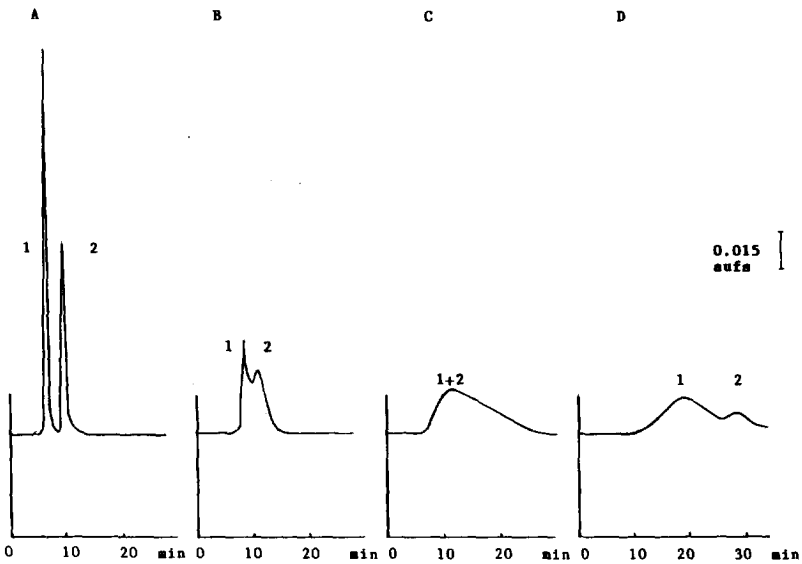

Fig. 5. RP-HPLC of (1) 20-hydroxyecdysone and (2) inokosterone. Column B; flow-rate, 1.0 ml/min. Eluent: (A) butanol–water (1:24, v/v); (B) as (A) but containing 0.02 *M* boronic acid; (C) as (A) but containing 0.1 *M* boronic acid; (D) as (A) but containing 0.3 *M* boronic acid.

shown for ecdysteroids under conditions of supercritical fluid chromatography [13]. Consequently, boronic acid affects the second participant in the process, the sorbent–solute interface, to a greater extent than product formation in the mobile phase. The reason for this may be a dynamic modification of the sorbent surface and subsequent reaction of ecdysteroids with a partially sorbed form of boronic acid. We cannot offer a detailed mechanism because it requires special experiments with sorbents of different surface densities of residual silanols, which is suggested to play a significant role in separations with boronic acid.

Specially prepared ecdysteroid mixtures were also subjected to chromatographic separation with butanolic eluents with different boronic acid contents. It is clear (Figs. 5 and 6) that the primary question regarding the absence or presence of a reactable diol in the molecule can be answered even during the above analysis without obtaining pure ecdysteroids.

4. Conclusion

One can use the above-described phenomenon in some practical cases. Boronic acid-containing eluents allow us to detect such structural elements of ecdysteroid molecules as *cis* vicinal diols and their combinations. Selectivity has been demonstrated towards the following positions: 20,22-diol and pairs of 1,2-, 24,25- and 25,26-diols with 20,22-diol.

The advantages of the above approach are its simplicity and rapidity when the most popular RP-HPLC is used with minor modification of eluents for resolving new kinds of experimental problems. Nevertheless, further study is required to establish the reason for the retardation of ecdysones in the presence of boronic acid and the role of different organic solvents in this process.

Reversed-phase affinity chromatography seems a convenient technique for preliminary investigation of the structure of new ecdysteroids

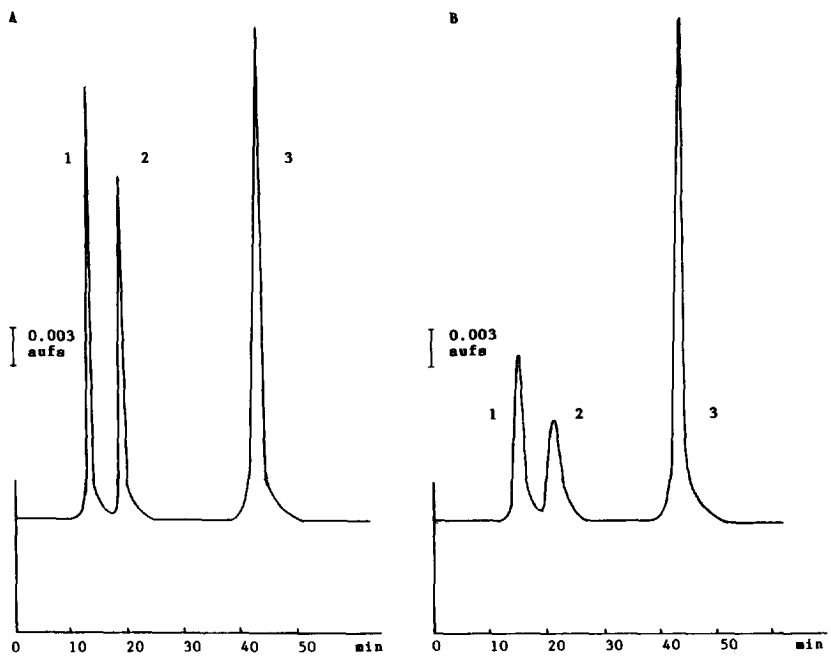

Fig. 6. RP-HPLC of a model mixture of (1) 20-hydroxyecdysone, (2) inokosterone and (3) ecdysone. Column C; flow-rate, 1.0 ml/min. Eluent: (A) butanol–water (1:24, v/v); (B) as (A) but containing 0.01 *M* boronic acid.

when no standards are available. Analysis may be undertaken with a range of ecdysteroids during one injection, which facilitates obtaining pure individual ecdysteroids.

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References

[1] A.A. Akhrem and N.V. Kovganko, Ecdysteroids: Chemistry and Biological Activity, Nauka i Tekhnika, Minsk, 1989.
[2] J. Koolman (Editor), Ecdysone: from Chemistry to Mode of Action, Georg Thieme, Stuttgart, 1989.
[3] T. Vaisar and J. Píš, Rapid Commun. Mass Spectrom., 7 (1993) 46–52.
[4] E.M. Schwarz, Interaction of Boronic Acid with Alcohols and Oxyacids, Zinatne, Riga, 1990.
[5] S.J. Murphy, E.D. Morgan and I.D. Wilson, in A.R. McCaffery and I.D. Wilson (Editors), Chromatography and Isolation of Insect Hormones and Pheromones, Plenum Press, New York, 1990, p. 131.
[6] R. Lafont, E.D. Morgan and I.D. Wilson, J. Chromatogr. A, 658 (1994) 31–53.
[7] J. Píš and J. Harmatha, J. Chromatogr., 596 (1992) 271–275.
[8] J. Píš, J. Hykl, T. Vaisar and J. Harmatha, J. Chromatogr. A, 658 (1994) 77–82.
[9] C.B. Reese, in J.F.W. McOmic (Editor), Protective Groups in Organic Chemistry, Plenum Press, London, 1973, and Mir, Moscow, 1976.
[10] D. Guedin-Vuong, Y. Nakatani, B. Luu and G. Ourisson, Tetrahedron Lett., 26 (1985) 5959–5962.
[11] D. Guedin-Vuong, Y. Nakatani and G. Ourisson, Croat. Chem. Acta, 58 (1985) 547–557.
[12] I.D. Wilson, J. Planar Chromatogr., 5 (1992) 316–318.
[13] J.-H. Shim, I.D. Wilson and E.D. Morgan, J. Chromatogr., 639 (1993) 281–285.
[14] J. Píš, J. Hykl, M. Budesinsky and J. Harmatha, Collect. Czech. Chem. Commun., 58 (1993) 612–618.