Application of Borate Ion-Exchange Mode High-Performance Liquid Chromatography to Separation of Glycosides: Saponins of Ginseng, Sapindus mukurossi GAERTN. and Anemone rivularis BUCH.-HAM.

HIROYUKI YAMAGUCHI,* a HIROMICHI MATSUURA, a RYOJI KASAI, b KENJI MIZUTANI, b HIROKO FUJINO (née KIMATA), b KAZUHIRO OHTANI, b TORU FUWA a and OSAMU TANAKA b

Central Research Laboratories, Wakunaga Pharmaceutical Co., Ltd., a Shimokotachi 1624, Koda-cho, Takata-gun, Hiroshima 729-64, Japan, Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, b Kasumi, Minami-ku, Hiroshima 734, Japan

(Received January 20, 1986)

High-performance liquid chromatography (HPLC) of borate complexes on a basic ion-exchange column was investigated for the separation of saponins of Ginseng, Sapindus mukurossi and Anemone rivularis, as well as other synthetic glycosides including steviol glycosides. It was found that HPLC in this mode is effective for analysis and preparative separation of a variety of glycosides, especially isomeric glycosides, containing xylpyranosyl, arabinofuranosyl or arabinopyranosyl units.

Keywords—HPLC; borate complex; ion-exchange column; separative analysis; glycoside; saponin; Ginseng; Sapindus mukurossi; Anemone rivularis; steviol glycoside

In connection with studies on physiologically active natural glycosides, the separative analysis of glycosides by high-performance liquid chromatography (HPLC) on a reversed-phase column or silica gel column has been investigated extensively. However, HPLC of glycosides on silica gel generally requires a mixture of three solvents as a mobile phase i.e., CHCl3–CH3OH–H2O, EtOAc–BuOH–H2O, etc., which is unfavorable for sensitive peak detection by ultraviolet (UV) absorption measurement at short wavelength. Further, it is undesirable to use chlorine-containing solvents in preparative-scale HPLC because of the problem of disposal without causing environmental pollution. In reversed-phase HPLC, the peak resolution between isomeric glycosides which differ from each other only in an aldohexosyl or aldopentosyl unit is not always sufficient. For instance, saponins, 1, 2 and 3 (Chart 1) from pericarps of Sapindus mukurossi GAERTN. (Japanese name: Enmeihi), 1) which are isomers of the terminal aldopentosyl unit, are hardly separated by HPLC on a reversed-phase column.

It is known that certain neutral polyhydroxy compounds react with borate ion to form borate complexes which are negatively charged ions. The formation of a borate complex of such a type is of particular value in the chromatographic separations of carbohydrates. Neutral mono- and oligosaccharides can be separated by HPLC on ion-exchange resins in the presence of borate ions (borate ion-exchange mode). 2) In the present paper, the application of HPLC in this mode to the separation of certain glycosides is described.
Experimental

Materials—Ginsenosides-Rb₁ (4), -Rb₂ (5), -Rb₃ (6), -Re (7), -Rd (8), -Re (9) and -Rg₁ (10) were separated from Ginseng roots. Saponins 1, 2 and 3 were obtained from Enneihi and the saponin CP₄ (11) and huzhangosides-A (12), -B (13) and -C (14) were isolated from roots of Anemone rivularis BUCH.-HAM. (Hu-zhang-cao) as reported recently. Methyl α-L-arabinopyranoside (15), methyl α-L-arabinofuranoside (16), a mixture of methyl D-xylopyranoside and D-xylofuranoside, methyl 2-O-α-L-arabinopyranosyl-α-L-arabinopyranoside (17) and methyl 2-O-β-D-xylopyranosyl-α-L-arabinopyranoside (18) prepared for a nuclear magnetic resonance (NMR) study of arabinosides were used. Four sweet steviol glycosides (19—22) were synthesized from steviolmonoside (23) for a study on the structure—sweetness relationship, the details of which will be reported elsewhere.

Apparatus—A Jasco Trirotar-111 HPLC apparatus equipped with a Jasco Uvidec 100-IV variable-wavelength UV detector was used.

Selection of Column Packing—For analysis of mono- and oligosaccharides in the borate ion-exchange mode, a strongly basic ion-exchanger based on a microporous homogeneous matrix (polystyrene-divinylbenzene type) has generally been utilized. However, it was found that because of physical adsorption, oligoglycosides were hardly eluted in HPLC on a column of this type. Accordingly, an ion-exchanger which has a basic group, -N⁺HET₂, on a packing of polyvinyl alcohol type for gel filtration chromatography was used; Asahipak ES-502N (Asahikasei Kogyo Co., Ltd.); column size, 7.60 mm i.d. × 100 mm; ion-exchange capacity, 0.5 ± 0.1 meq/g. This packing is suitable for the present study due to its lower degree of swelling-shrinking as well as weaker adsorption, though the ion-exchange capacity is slightly less than that of the packing mentioned above.

![Chart 1](image)

![Chart 2](image)

Ara(p), α-L-arabinopyranosyl; Xyl, β-D-xylopyranosyl; Glc, β-D-glucopyranosyl; Rha, α-L-rhamnopyranosyl; Ara(f), α-L-arabinofuranosyl; Rib(p), β-D-ribofuranosyl; Gal, β-D-galactopyranosyl

Chart 2
Results and Discussion

Ginseng Dammarane Saponins

A number of physiologically active dammarane saponins have been isolated from Ginseng roots.3) For the purpose of chemical evaluation of this crude drug and its medicinal prescriptions, several papers on HPLC analysis of these saponins on reversed-phase columns7,8) or on a silica gel column9) have appeared in the literature. In this work, separation of the major saponins, 4, 5, 7—10 (Chart 1) by means of borate ino-exchange mode HPLC was investigated.

Effect of Solvent—Because of physical adsorption, these saponins were not eluted with aqueous borate solution, which has generally been used as a mobile phase for the separation of mono- and oligosaccharides by HPLC in this mode.2) Accordingly, acetonitrile (CH₃CN) which does not form a complex with borate, was added to the mobile phase. Under the conditions shown in Fig. 1, the correlation of concentration of CH₃CN with capacity ratio (k') of the saponins was explored; it was found that with 15% (v/v) aqueous CH₃CN, the separation of 4 and 7 was incomplete, while the use of 10% (v/v) aqueous CH₃CN resulted in

![Fig. 1. Effect of CH₃CN on the Capacity Ratio (k')](image1)

Column, Asahipak GS-502N; flow rate, 0.5 ml/min; column temp., 60°C.

![Fig. 2. Effect of H₂BO₃ Concentration on the Capacity Ratio (k')](image2)

Column conditions are the same as in Fig. 1.

![Fig. 3. Effect of Column Temperature on the Capacity Ratio (k')](image3)

Eluent, 0.25M H₂BO₃ in 12.5% (v/v) CH₃CN.
very slow elution of 8. The best chromatogram was obtained by using 12.5% aqueous CH$_3$CN as a solvent.

**Influence of Concentration of Boric Acid and Column Temperature**—It was found that the elution of saponins was accelerated as the concentration of boric acid or the column temperature was increased (Figs. 2 and 3). With regard to the separation of 4 and 7, increase of the concentration of boric acid reduced the resolution of peaks of 4 and 7, while increase of the column temperature resulted in improvement of the separation of the saponins.

Based on these results, the analysis was carried out by using 0.25 M borate solution in 12.5% (v/v) aqueous CH$_3$CN as a mobile phase at 70 ºC. As shown in Fig. 4, the chromatogram showed excellent separation of the saponins, being more effective than HPLC on a reversed-phase column; with the latter, it is difficult to separate all the major saponins at once (Fig. 5).

**Saponins of Pericarps of Enmeihi**

An oriental crude drug, Enmeihi, has recently been used as a source of natural surfactant rather than for medicinal purposes, and a number of saponins have been isolated from this crude drug (Chart 1). Of these saponins, the monodesmosides, 1, 2 and 3 are sparingly
soluble in water in the pure state, though the water solubilities of these monodesmosides were greatly increased by the co-occurring bisdesmosides.\textsuperscript{1,10} Further, it was found that solutions of these monodesmosides solubilized with the aid of the bisdesmosides produced a remarkable enhancement of the absorption of β-lactam type antibiotics from rat intestine and rectum.\textsuperscript{11} As already mentioned, these monodesmosides differ from each other in the terminal aldopentosyl unit (1, α-L-arabinopyranosyl unit; 2, β-D-xylopyranosyl unit; 3, α-L-arabinofuranosyl unit) and the separation of 1–3 could not be accomplished by reversed-phase HPLC.

The effects of concentrations of borate and CH$_3$CN on $k'$ in the borate ion-exchange mode HPLC of 1–3, were studied.

As shown in Fig. 6, increase of molarity of borate led to decrease of the retention time as well as better separation of 2 and 3, whereas it resulted in worse resolution of the peaks of 1 and 3. Increase of concentration of CH$_3$CN resulted in faster elution of each monodesmoside and worse separation of 2 and 3. The effect of pH on $k'$ is shown in Fig. 7. Increase of pH led to worse separation and decrease of the retention time, and at pH 9.3, sufficient separation of the three monodesmosides could not be obtained. This is presumably due to the decrease of

![Fig. 6. Effect of CH$_3$CN and H$_3$BO$_3$ Concentrations on the Capacity Ratio ($k'$)](image)

- pH=8.50 (adjusted with 5N NaOH); ---, 20% CH$_3$CN; ----, 30% CH$_3$CN. The other conditions are the same as in Fig. 1.

![Fig. 7. Effect of pH on the Capacity Ratio ($k'$)](image)

Eluent, 0.4M H$_3$BO$_3$ in 20% (v/v) CH$_3$CN. The other conditions are the same as in Fig. 1.

![Fig. 8. Chromatogram of Saponins of Sapindus mukausi GAERTN.](image)

(A) standard mixtures, (B) extract of Sapindus mukausi GAERTN. Eluent, 0.4M H$_3$BO$_3$ in 20% (v/v) CH$_3$CN; pH=8.00 (adjusted with 5N NaOH); flow rate, 0.5 ml/min; column temp., 75°C; chart speed, 2.5 mm/min.
capacity of the exchanger.

Based on these results coupled with the effect of column temperature, good separation of each saponin was obtained under the following conditions (Fig. 8): mobile phase, 0.4 M boric acid solution in 20% (v/v) aqueous CH$_3$CN; pH 8.00; column temperature, 75 °C.

Saponins of Roots of *Anemone rivularis* BUCH.-HAM. (Chinese Name: Hu-zhang-cao)

Several saponins have been isolated from this Chinese medicinal plant, as shown in Chart 2. The monodesmosides, saponin CP$_4$ (11) and huzhangoside-A (12), differ from each other only in the inner glycosyl unit (11, α-L-arabinopyranosyl unit; 12, β-D-xylpyranosyl unit) and effective separation of these monodesmosides by HPLC has been difficult.

Since the aglycone of both 11 and 12 is oleanolic acid (24), which is less polar than that (hederagenin (25)) of enmeihi-saponins, 1—3, the separation of 11 and 12 by HPLC in the borate ion-exchange mode was accomplished by using a mobile phase with a higher concentration (30% v/v) of CH$_3$CN than in the case of 1—3. The relation of borate concentration to $k'$ was also investigated (Fig. 9). Based on these results, excellent separation of 11 and 12 was obtained by using 0.3 M borate solution in 30% (v/v) CH$_3$CN as the mobile phase, as shown in Fig. 10. The isomeric saponins, huzhangosides B (13) and C (14), from the same crude drug are bisdesmosides corresponding to the monodesmosides, 11 and 12, respectively, (Chart 2). Complete separation of these bisdesmosides by HPLC in the borate ion-exchange mode was also obtained under the conditions shown in Fig. 10.

Relationship between Structure and Elution Order

The results (elution order) of the present study are summarized in Table 1 in comparison...
TABLE I. Comparison of Elution Order of Glycosides

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds</th>
<th>Elution order (A)</th>
<th>Elution order (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Ginsenoside-Rb₁</td>
<td>-Glc⁶Glc</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Ginsenoside-Rb₃</td>
<td>-Glc⁶Xyl</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>Ginsenoside-Re</td>
<td>-Glc⁶Ara(f)</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>Ginsenoside-Rb₂</td>
<td>-Glc⁶Ara(p)</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Sapindoside-B</td>
<td>-Ara(p)²Rha³Xyl</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Saponin-C</td>
<td>-Ara(p)²Rha³Ara(f)</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>Saponin-A</td>
<td>-Ara(p)²Rha³Ara(p)</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>Huzhangoside-A</td>
<td>-Xyl²Rha³Rib(p)</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>CP₄</td>
<td>-Ara(p)²Rha³Rib(p)</td>
<td>2</td>
</tr>
<tr>
<td>14</td>
<td>Huzhangoside-C</td>
<td>-Xyl²Rha³Rib(p)</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>Huzhangoside-B</td>
<td>-Ara(p)²Rha³Rib(p)</td>
<td>2</td>
</tr>
</tbody>
</table>

Methyl monosaccharides

| 16  | Me-O–Ara(f)               | 1                 | 1                 | Fig. 12 |
| 15  | Me-O–Ara(p)               | 2                 | 2                 |

Methyl disaccharides

| 18  | Me-O–Ara(p)²Xyl           | 1                 | 2                 | Fig. 13 |
| 17  | Me-O–Ara(p)²Ara(p)        | 2                 | 1                 |

Steviol glycosides

| 19  | Glc ester                 | -COO–Glc           | 1                 | 2                 | Fig. 14 |
| 20  | Xyl ester                 | -COO–Xyl           | 2                 | 4                 |
| 21  | Gal ester                 | -COO–Gal           | 3                 | 1                 |
| 22  | Ara(p) ester              | -COO–Ara(p)        | 4                 | 3                 |

<sup>a</sup> Can not be observed as separated peaks. (A): on a borate-anion exchange column (Asahipak ES-502N). (B): on a reversed-phase column (Radialpak-βBondapak C18). Abbreviations of sugars: see Chart 2.

Fig. 11. Chromatogram of Ginseng Saponins
Eluent, 0.1 M H₂BO₃ in 10% (v/v) CH₃CN; column temp., 60°C.

Fig. 12. Chromatogram of Methyl Monosaccharides (α-L-Arabinosides)
Eluent, 0.1 M H₂BO₃; column temp., 50°C.

with those of reversed-phase HPLC. A significant relationship between the elution order and the composition of the sugar moiety was found for each series of isomeric oligoglycosides. In the borate ion-exchange mode HPLC, the isomeric saponins of Ginseng, Enmeihi and Huzhang-cao were eluted in the order of β-D-xylpyranoside, α-L-arabinofuranoside and α-L-arabinopyranoside (Ginseng saponins 6—7—5 (Fig. 11), enmeihi-saponins 2—3—1 (Fig. 8).
and Hu-zhang-cao saponins 12—11 and 14—13 (Fig. 10)). This elution order can be explained in terms of the structural preference for formation of the borate complex. The α-L-arabinopyranoside unit has a pair of cis-hydroxyls at the 3,4-positions, which is favorable for formation of the complex. On the other hand, the β-D-xylopyranoside unit has no cis-1,2-diol moiety. The α-L-arabinofuranoside unit also has no cis-1,2-diol system, but can form a borate complex between the 2- and 5-hydroxyl groups.

The same elution order was observed for isomeric pairs of some synthetic glycosides; methyl α-L-arabinofuranoside (16)—methyl α-L-arabinopyranoside (15) (Fig. 12), methyl 2-O-β-D-xylopyranosyl-α-L-arabinopyranoside (18)—methyl 2-O-α-L-arabinopyranosyl-α-L-arabinopyranoside (17) (Fig. 13) and β-D-xylopyranosyl ester of steviolmonoside (20)—α-L-arabinopyranosyl ester of steviolmonoside (22) (Fig. 14). It was also found that in the borate ion-exchange mode HPLC, β-D-galactopyranosyl ester of steviolmonoside (21) was eluted more slowly than the corresponding β-D-glucopyranosyl ester (19) and β-D-xylopyranosyl ester (20) (Fig. 14) because of the favorable structure of galactopyranosyl unit for formation of a borate complex (presence of the 3,4-cis-diol system). Methyl-D-xylofuranoside (both α- and β-anomers) was eluted more slowly than methyl D-xylopyranoside (a mixture of α- and β-anomers) (Fig. 15). This is presumably because of the formation of the borate complex at the 3- and 5-hydroxyl groups in xylofuranoside.

Since aqueous CH₃CN is used as a mobile phase in the present study, the reversed-phase partition mode is also implicated in the elution order; the β-D-glucopyranosyl isomer (4) is eluted faster than the corresponding β-D-xylopyranosyl isomer (6) (Fig. 11). In the series of glycosyl esters of steviolmonoside (23), β-D-glucopyranosyl (19) and β-D-galactopyranosyl (21) esters are eluted before β-D-xylopyranosyl (20) and α-L-arabinopyranosyl (22) esters, respectively (Fig. 14).

Borate can be easily removed as volatile methyl borate by repeated co-distillation of each eluate with methanol, so that the present procedure is promising not only for analysis but also for the preparative separation of glycosides.
References

6) M. Darise, K. Mizutani, R. Kasai and O. Tanaka, to be published.