Pharmacochemistry

HPLC Analysis of Poly[3-(3,4-Dihydroxyphenyl)glyceric acid] Preparations from Symphytum asperum and Anchusa italic a (Boraginaceae) Using Different Gel-Filtration Columns


* Tbilisi State Medical University I.Kutateladze Institute of Pharmacochemistry, Tbilisi, Georgia,
** Phenomenex, Inc., R&D Group, Technology and Media Development, Torrance, CA, USA

(Submitted by Academy Member Bezhan Chankvetadze)

ABSTRACT. The main chemical constituent of high-molecular preparations from Symphytum asperum and Anchusa italic a, poly[3-(3,4-dihydroxyphenyl)glyceric acid] (PDPGA), according to high-performance liquid chromatography (HPLC) gel-filtration chromatography (GFC) analysis is not covalently bound to residual polysaccharides. It is rather difficult to completely separate the polysaccharides from PDPGA by HPLC (GFC). This phenomenon can be explained as due to the presence of manifold hydrogen bonds between the polysaccharides and PDPGA. It will hold the residual polysaccharides together with the phenolic polymer during fractionation by HPLC (GFC). The PDPGA supposedly can be formed with polysaccharides a complex macromolecular architecture up to their supramolecular organization.


Key words: symphytum asperum, anchusa italic a, poly[3-(3,4-dihydroxyphenyl)glyceric acid], HPLC, gel-filtration chromatography (GFC).

Previously we reported on the isolation of water-soluble high-molecular preparations (HMPs) by ultrafiltration on membrane filters of crude polysaccharides from Symphytum asperum (HMP-SA), S. caucasicum (HMP-SC), S. officinale (HMP-SO) and Anchusa italic a (HMP-AI) [1-3]. Fractionation by ultrafiltration removed the majority of ballast polysaccharides, but the obtained preparations contained some residual polysaccharides. According to IR and NMR spectroscopy data, the main chemical constituent of these fractions is either poly[3-(3,4-dihydroxyphenyl)glyceric acid] (PDPGA) or poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene] [2-7] (Fig. 1).

This compound is one of the first representatives of natural polyethers with a residue of 3-(3,4-dihydroxyphenyl)glyceric acid as the repeating unit. It showed strong anticomplementary, antioxidant and
anti-inflammatory activities [1,2,8,9]. Then we tried to remove the residual polysaccharides and to purify HMP-SA and HMP-SO or clarify the structure significance of polysaccharides. The gel-filtration chromatography (GFC) on Sepharose 2B column of both preparations showed two polysaccharides elution peaks and phenolic polymer PDPGA elution plot. The polysaccharides peaks did not coincide with PDPGA peak showing some shifts from it. One polysaccharide peak overlapped the beginning and the other one the end of PDPGA elution curve [1,2]. According to these data PDPGA apparently is not covalently bound to the polysaccharides. However, we did not get a close-cut separation of residual polysaccharides from PDPGA during GFC on Sepharose 2B column. In the current study, besides the coincidence of IR and NMR spectra of PDPGA for HMP-SA, HMP-SC, HMP-SO and HMP-AI [2-7], we found also close similarity of their circular dichroism (CD) spectra.

The CD spectra of HMP-SA, HMP-SC, HMP-SO and HMP-AI have similar profiles and showed at the same wavelengths positive (194, 214, 280, 286 nm) and negative (204, 236 nm) Cotton effects (Fig. 2). These data confirmed that two chiral carbon atoms of PDPGA (Fig. 1) of HMP-SA, HMP-SC, HMP-SO and HMP-AI have one and the same absolute configuration. However, the establishment of the absolute configuration of these chiral atoms will be the subject of further research.

Within our ongoing research for purification of different HMPs from residual polysaccharides, the main subject of current work was the attempt to fractionate HMP-SA and HMP-AI by HPLC on two types of GFC columns - silica-based Biosep 4000 and polymer-based Polysep 2000 and 6000 with use of both UV and RI detectors (Figs. 3.1-3.6).

In our previous work the absorption maxima of HMP-SA, HMP-SC, HMP-SO and HMP-AI at 286, 282-280 (shoulder) and 252 nm in veronal-saline buffer, pH 7.35, were observed in the UV spectra of these preparations [1-3]. Sugars have no chromophores and therefore do not absorb light in the UV range. Consequently, this absorption could arise from PDPGA and we can detect it by HPLC analysis of HMP-SA and HMP-AI using UV detector (Fig. 3), but at the same time we cannot detect polysaccharides in this preparation by UV detector. Therefore, in order to detect both PDPGA and residual polysaccharides we carried out HPLC analysis of HMP-SA and HMP-AI using RI detector (Figs. 4-7).

At 280 nm and 286 nm of UV detector, only the high molecular weight part of both S.asperum polymer and A.italica polymer showed up and the low MW part of the polymers did not show up. When wavelength was set at 252 nm, the low molecular weight part of the polymers showed up besides the high MW part of the polymers (Fig. 3).

On RI detector, we can see that both polymers have high MW parts (Fig. 8), which according to calibration of column by mixture of standard proteins...
(Fig. 9) are more than 669 KDa and supposedly might be phenolic polymer PDPGA. Relatively lower MW parts (Fig. 8) around 150 KDa, that we could not see on UV detector, presumably represent polysaccharides.

Thus, we did not get a clear separation of residual polysaccharides from PDPGA by HPLC analysis of HMP-SA and HMP-AI on column Biosep 4000 (Figs. 4, 8). HPLC analysis of HMP-SA on column Polysep 2000 (Fig. 5) showed bad separation, while the column Polysep 6000 gave some separation (injection 20 ul) (Fig. 6). The best separation of PDPGA and residual polysaccharides, unlike the column Polysep 2000 (Fig. 5), was achieved on the column Polysep 6000 (injection 9 ul) (Fig. 7). In this case the major peak (Fig. 6) split up into two peaks (Fig. 7). The first and the third peaks probably belong to polysaccharides and the second one to PDPGA.

Thus, chromatographic profiles of HMP-SA on Sepharose 2B GFC column [1,2] and HPLC on GFC column Polysep 6000 (Fig. 7) were similar, but separation by HPLC on Polysep 6000 was better. According to these data we confirmed our previous supposition that polysaccharides contents of HMP-SA are not
covalently bounded with PDPGA. However, it is very difficult to completely separate the polysaccharides from PDPGA by GFC. This phenomenon can be explained due to the presence of hydrogen bonds between PDPGA and residual polysaccharides which will hold the polysaccharides together with the phenolic polymer during fractionation by ultrafiltration and GFC. The PDPGA is chemically simple, but its molecules can form with each other and with the molecules of residual polysaccharides complex macromolecular associates up to their supramolecular organization due to hydrogen bonds [10].

The existence of non-covalently bonded organized supramolecular self-assembly comprising molecules of PDPGA and residual polysaccharides will be of interest for further research. Supramolecular architecture of this system is of great interest due to their hierarchical ordered structures (e.g., secondary conformations) and due to their potential biomedical and pharmaceutical applications [6,8,9,11-13].

In our future study we will try to carry out HPLC analysis of HMP-SA and HMP-AI using DMSO or 6M urea [14] as mobile phases to destroy hypothetical inter-molecular hydrogen bonds.

**Experimental**

**Extraction and Isolation.** Hot water extraction of crude polysaccharides from grounded and pretreated in Soxhlet apparatus plant materials was carried out as described in paper [15]. Further fractionation in stirred ultrafiltration cell on membrane filters afforded HMP-SA, HMP-SC, HMP-SO and HMP-AI [1-3].

**CD spectra.** CD spectra of HMP-SA, HMP-SC, HMP-SO and HMP-AI were performed on a Jasco J-715 instrument (Jasco Co, Tokyo, Japan) equipped with peltier temperature control system. CD spectrum of HMP-SA (C = 0.12 mg/ml, H2O): \( \Delta \varepsilon_{286} + 3 \), \( \Delta \varepsilon_{280} + 2.5 \), \( \Delta \varepsilon_{255.6} - 2.5 \), \( \Delta \varepsilon_{214} + 48 \), \( \Delta \varepsilon_{203.6} - 26 \), \( \Delta \varepsilon_{193.6} + 3.5 \).

**HPLC analyses.** In the case of data on UV detector, HPLC separations of HMP-SA and HMP-AI were performed on Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) with quad pumps, auto sampler and VWD detector. Chemstation software was used for data analysis. In the case of
data on RI detector, Shimadzu HPLC SCL-10A VP system was used (Shimadzu Scientific Instruments, Somerset, NJ, USA) with LC-10AP pump and RID-10A RI detector and Thermasphere column heater TS-130. EZstart 7.4 SP1 software was used for data analysis.

Test conditions:

a) silica-based GFC column Biosep 4000 (300 x 7.8 mm) SN517561-4, (Phenomenex Inc., Torrance, CA, USA); mobile phase - 100 mM sodium phosphate buffer pH 6.8 + 0.025% NaN₃; flow – 1 ml/min; detection - UV 286, 280 and 252 nm; temp. – ambient; injection - 5 µl; sample - 20 mg HMP-SA and HMP-AI was dissolved in 1mL 100 mM sodium phosphate buffer pH 6.8.

b) silica-based GFC column Biosep 4000 SN517561-4 and polymer-based GFC columns Polysep 2000 and Polysep 6000 (33 x 7.8 mm) (Phenomenex Inc., Torrance, CA, USA); mobile phase – water; detection – RI; flow - 1ml/min; temp. – ambient or 60°C; injection - 20 ul or 9 ul; sample - 10 mg of HMP-SA and HMP-AI was dissolved in 1.5 mL 30 mM sodium phosphate buffer pH 6.8.

c) the molecular weights of HMP-SA and HMP-AI were estimated by calibration of the column using QC standard proteins mix test on GFC 4000 5um. Test conditions: mobile phase - 100 mM sodium phosphate buffer pH 6.8 + 0.025% NaN₃; flow - 1ml / min; detection - UV 280nm; temp. – ambient; injection - 5µl; samples - thyroglobulin (MW 669,000) – 7.774 min., IgA (MW 300,000) – 8.586 min., IgG (MW 150,000) – 9.532 min., ovalbumin (MW 44,000) – 10.235 min., myoglobin (MW 17,000) – 10.932 min and uridine (MW 244) – 11.958 min.

**Symphytum asperum-ის და Anchusa italica-ს (Boraginaceae) ჰიდრო [3-(3,4-დიჰიდროქინოფენილ)გლიცერინი] პრეპარატების პრეპარატები (HPLC) ანალიზი და ფარმაკოგიმიური პროპერთები**

* არიან ყველა თავის სახელწოდება, და პირველი კონტაქტი, არიან ესტში იმუშავება, არიან შემდგომ ტექნიკური, და არიან ეთიკური, გამჭვირვალური, სოფლები, კოოპერაციული, და სხვა

(შინაგანი მოწყობილობები პირველი კონტაქტი საჭირო არ არის)

**Symphytum asperum-ის და Anchusa italica-ს ჰიდრო [3-(3,4-დიჰიდროქინოფენილ)გლიცერინი] პრეპარატები (HPLC) ანალიზი და ფარმაკოგიმური პროპერთები**

REFERENCES


Received December, 2012