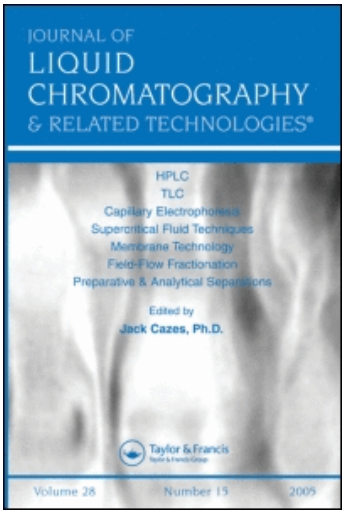


**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Analysis of Antineoplastic Polysaccharides from *Mycobacterium Bovis* BCG Vaccine by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection

Ronghua Wang[a]; Melvin E. Klegerman[a]; Michael J. Groves[a]
[a] Institute for Tuberculosis Research College of Pharmacy University of Illinois at Chicago, Chicago, IL

**To cite this Article** Wang, Ronghua , Klegerman, Melvin E. and Groves, Michael J.(1996) 'Analysis of Antineoplastic Polysaccharides from *Mycobacterium Bovis* BCG Vaccine by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection', Journal of Liquid Chromatography & Related Technologies, 19: 16, 2691 — 2698
**To link to this Article: DOI:** 10.1080/10826079608014049
**URL:** http://dx.doi.org/10.1080/10826079608014049

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2691–2698 (1996)

# ANALYSIS OF ANTINEOPLASTIC POLYSACCHARIDES FROM *MYCOBACTERIUM BOVIS* BCG VACCINE BY HIGH PERFORMANCE ANION EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION

Ronghua Wang, Melvin E. Klegerman,
Michael J. Groves*

Institute for Tuberculosis Research
College of Pharmacy
University of Illinois at Chicago (M/C 964)
950 S. Halsted St.
Chicago, IL 60607



## ABSTRACT

Aqueous extracts of *Mycobacterium bovis* BCG vaccine, Tice® substrain, showed potent antineoplastic activity against a murine S180 sarcoma cell line in vivo. Following Sephadex LH-20 chromatography, one fraction (PS1A) was found to have antineoplastic activity, and contains complex polysaccharides. PS1A was further separated into four subfractions by Sephadex G-75 column chromatography. An HPLC method has been established for the analyses of polysaccharides in PS1A and its subfractions. Using a Dionex DX500 HPLC system, separation was achieved on a CarboPac PA1 anion exchange column by gradient elution with 0.1 M NaOH and 0.9 M NaOH/1 M NaOAc. Pulsed amperometry with three potential waveforms (E1=0.1 v, E2=0.7v and E3=-0.1v) was used for detection.

The HPLC chromatogram of PS1A showed three major peaks with retention times of 8.5, 15.5 and 19.25 min., respectively, and these three peaks have been identified in the subfractions of PS1A.

## INTRODUCTION

Attenuated *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) has been used as a tuberculosis vaccine for more than 70 years. In 1990, it was approved by the U.S. Food and Drug Administration as an immunotherapeutic agent for the treatment of superficial bladder cancer.[1] A boiling water extract of BCG, termed PS1, showed antineoplastic activity in the murine S180 sarcoma model in vivo.[2] The antineoplastic components of PS1 have been separated by chromatography using both Sephadex LH-20 and Sephadex G-75 columns, and have been shown to be high molecular weight glycans.[3] In order to develop a therapeutic agent from PS1, its production, formulation and metabolism need to be studied. However, before doing any of these functions, it is imperative to develop a rapid and accurate method for the analysis of these antineoplastic polysaccharides.



High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been developed recently for the analysis of carbohydrates.[4] At high pH, normally 11-13, the hydroxyl groups of carbohydrates dissociate and become negatively charged, allowing separation by anion exchange chromatography. Pulsed amperometry is more sensitive and selective than either ultraviolet spectrophotometry or refractometry, and can detect picomolar quantities of monosaccharides. HPAEC-PAD has been used for the analysis of monosaccharides, oligosaccharides and polysaccharides. For example, polysaccharides with molecular weights up to 9 kDa have been analyzed using this method.[5] We report here the separation of antineoplastic polysaccharides from BCG using this technique.

## MATERIALS AND METHODS

Sodium hydroxide solution (50% w/w), phenol (white loose crystals), sulfuric acid, and acetic acid (glacial) were all purchased from Fisher Scientific (Itasca, IL) and sodium acetate trihydrate from Fluka Chemika (Ronkonkoma, NY). Antineoplastic polysaccharide samples were prepared from freeze-dried ampoules of *Mycobacterium bovis* BCG vaccine, Tice® substrain (lot 105 x 2), previously prepared at this Institute and stored at -20°C. Deionized, distilled water was used for the preparation of HPLC eluents.

### Fractionation of PS1

The preparation and fractionation of PS1 have been described previously.[3] Briefly, a solution of PS1, a boiling water extract of BCG, was applied to a Sephadex LH-20 column (Pharmacia Fine Chemicals, Piscataway, NJ), the column eluted with distilled water, and the eluent assayed for carbohydrates by a phenol/sulfuric acid method.[6] The antineoplastic activity of the resultant fractions was determined with a quantitative murine S180 sarcoma assay previously described.[7]

The active fraction, termed PS1A, was applied to a Sephadex G-75 column, and the resultant fractions were also assayed by the phenol/sulfuric acid method and the S180 sarcoma animal model. Animal test data are not reported here. Identified fractions had antineoplastic activity, although to varying degrees.



### Analysis of PS1A and its Subfractions with HPAEC-PAD

The HPAEC-PAD method was developed on a Dionex (Sunnyvale, CA) DX500 HPLC system which includes a GP40 quaternary gradient pump, a CarboPac PA1 anion exchange column (4x250 mm), and an ED40 electrochemical detector (gold working electrode). The system was interfaced with a Peaknet chromatography work station. All eluents were degassed, and the flow rate was 1 mL/min.

Samples of PS1A and its subfractions (mg/mL) were dissolved in deionized, distilled water, centrifuged at 10,000 rpm in a microcentrifuge (Fisher Scientific, model 59A), and the supernatant used for the subsequent HPLC analyses.

## RESULTS AND DISCUSSION

PS1 was separated into three fractions, designated PS1A, B and C, with the Sephadex LH-20 column. The antineoplastic fraction determined by S180 sarcoma assay, PS1A, was further separated into four fractions with the Sephadex G-75 column, designated PS1A1 through 4.

Separation and detection conditions of the antineoplastic polysaccharides were studied. It was found that the gradient elution program (Table 1) with solvents (a) 0.1 M NaOH and (b) 0.9 M NaOH/1 M NaOAc readily separated polysaccharide components. The waveform of the electrochemical detector for the analysis of polysaccharides is shown in Table 2.

**Table 1**

**GP40 Gradient Program A: 0.1M NaOH; B: 0.1M NaOH/0.9 M NaOAc**

| Time (min.) | Flow (mL/min) | %A | %B |
|---|---|---|---|
| Init. | 1 | 90 | 10 |
| 3 | 1 | 90 | 10 |
| 20 | 1 | 30 | 70 |
| 25 | 1 | 30 | 70 |
| 30 | 1 | 90 | 10 |

**Table 2**

**ED40 Detector Waveform**

| Time (s) | Potential (v) | Integration |
|---|---|---|
| 0.00 | 0.1 | |
| 0.20 | 0.1 | begin |
| 0.40 | 0.1 | end |
| 0.41 | 0.7 | |
| 0.60 | 0.7 | |
| 0.61 | -0.1 | |
| 1.0 | -0.1 | |



Using the established procedure, PS1A and its subfractions were analyzed. Figure 1 shows the HPLC chromatogram of PS1A, with three major peaks at retention times of 8.5, 15.5 and 19.25 min., respectively.

Figure 2 is the HPLC chromatogram of PS1A1, which showed a major peak with a retention time of 15.5 min. PS1A1 was characterized as a glucan with a dominant α-1,6 linkage.[3] Figure 3 is the HPLC chromatogram of PS1A2, which showed two peaks with retention times of 15.6 (PS1A2.1) and 19.25 min. (PS1A2.2). Although the molecular weights of PS1A1 and PS1A2.1 were different, 70 kDa and 20 kDa, respectively,[3] they behaved similarly on the anion exchange column. Accordingly, the retention times of PS1A1 and PS1A2.1 were similar, and these two components overlapped on the HPLC chromatogram of PS1A. On the other hand, PS1A2.1 and PS1A2.2

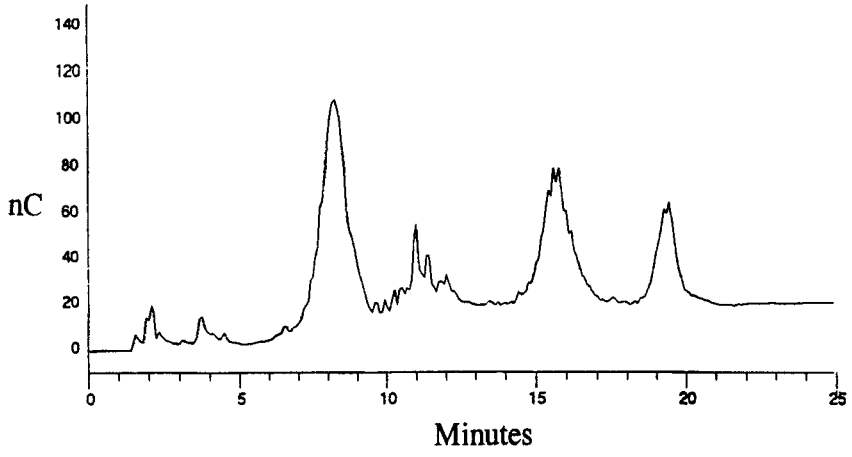


**Figure 1.** The HPLC chromatogram of PS1A. Conditions: CarboPack PA1 column; gradient elution with 0.1 M NaOH and 0.1 M NaOH/0.9 M NaOAc; flow rate, 1 mL/min.; detector, ED 40 electrochemical detector, pulsed amperometric.



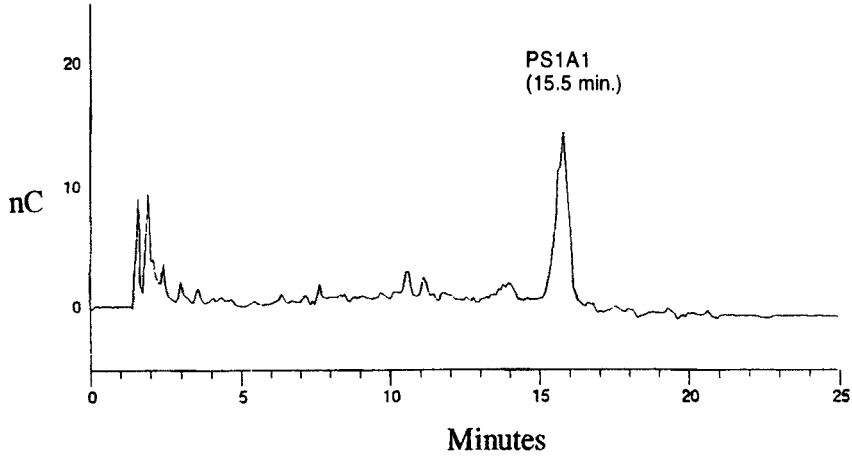


**Figure 2.** The HPLC chromatogram of PS1A1. Conditions: same as Figure 1.

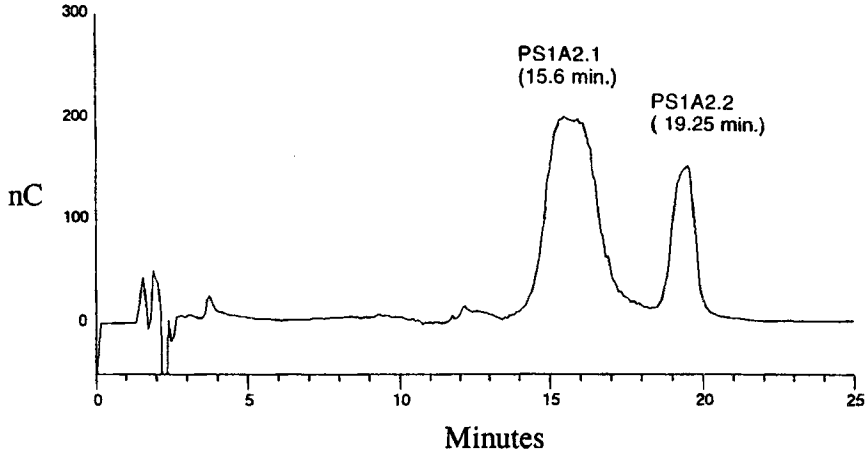


**Figure 3**. The HPLC chromatogram of PS1A2. Conditions: same as Figure 1.

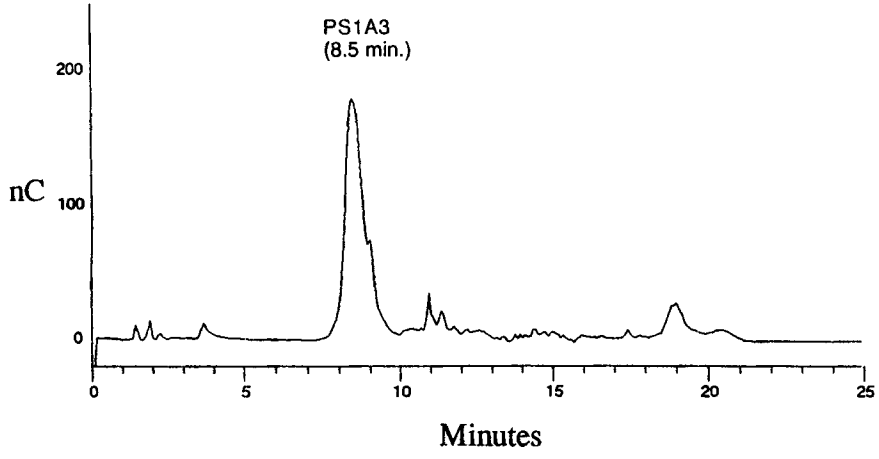


**Figure 4**. The HPLC chromatogram of PS1A3. Conditions: same as Figure 1.

had similar molecular weights, but behaved differently on the ion exchange column, and were easily separated. It is known that PS1A2.1 and PS1A2.2 are chemically different: the former is a arabinomannan, the latter is an arabinogalactan (unpublished results).

Figure 4 is the HPLC chromatogram of PS1A3, demonstrating one major peak with a retention time of 8.5 min. Monosaccharide compositional analysis showed that PS1A3 contained glucose, arabinose, galactose and an unidentified sugar (unpublished data).

## CONCLUSION

An HPAEC-PAD method has been developed on a Dionex DX500 HPLC system for the analysis of antineoplastic polysaccharides from BCG.

PS1 sub-fractions, PS1A and PS1A1-4, were analyzed by this method, and major peaks corresponding to these subfractions were identified on their HPLC chromatograms.



## ACKNOWLEDGEMENT

We thank Ms. Afeng Li for the preparation of PS1 and its subfractions.

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Received February 18, 1996
Accepted March 18, 1996
Manuscript 4084