SEPARATION OF PHENOLIC GLYCOLIPIDS IN MYCOBACTERIUM BOVIS BCG BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

MONA SALIMI, LADAN POURABDI*, MARYAM ZAKERI AND NAZANIN ABDIPOUR**

Research & Development Department, Pasteur Institute of Iran, Karaj, Iran,
**Organic Chemistry Department, Faculty of Chemistry, Sharif University, Tehran, Iran,*
***Biotechnology Department, Pasteur Institute of Iran, Tehran, Iran*

ABSTRACT

A crude phenolic glycolipid extract from *Mycobacterium bovis* BCG was fractionated by column chromatography. A reversed-phase high performance liquid chromatography (HPLC) method with UV detection at 275nm was developed for simultaneous detection and separation of phenolic glycolipids (PGLs) in *Mycobacterium bovis* BCG. This analysis provides a good resolution. Different solvent systems and columns for HPLC were compared. A system composed of acetonitrile-water in the ratio of 0→80% at a flow rate of 0.8 mL/min and C8 analytical column were found to be optimum for HPLC of the phenolic glycolipids. This simple method is therefore appropriate to purify these compounds present in *M. bovis* extract.

Keywords: HPLC-UV, mycobacterium, phenolic glycolipid.

INTRODUCTION

In microbial pathogenicity, mycobacteria play a predominant role as the etiologic agents of leprosy and tuberculosis (Wolinsky, 1984). The renewed interest in the detection, purification, and structural elucidation of the phenolic glycolipids of pathogenic mycobacteria prompted recent advances in the field of their immunological properties (Gaylord *et al*., 1987). The specific antigen of *Mycobacterium leprae* was characterized as phenolic glycolipid by Brennan and co-workers (Hunter *et al*., 1982) that formed by a unique trisaccharide consisting of 3,6-di-O-Me-β-D-Glcp-(1→4)-2,3-di-O-Me-α-L-Rhap-(1→2)-3-O-Me-α-L-Rhap. It is successfully used as serologic marker for the screening of lepromatous patients (Menzel *et al*., 1987, Fujiwara *et al*., 1984). It also seemed to be involved in the absence of cell-mediated immunity response by inducing suppressor lymphocytes which could inhibit the reactivity helper T-cell clones in lepromatous patients (Wolinsky, 1984, Mehra *et al*., 1984, Prasad *et al*., 1987). Moreover, it was reported that this glycolipid may protect *M. leprae* from the antimicrobial activity of activated human macrophages (Neill *et al*., 1988).

A related phenolic glycolipid was isolated from *M. tuberculosis* strain Canetti. Its oligosaccharidic moiety consists of trisaccharidic structure is 2,3,4-tri-O-Me-α-L-Fucp-(1→3)-α-L-Rhap-(1→3)-2-O-Me-α-L-Rhap (Daffe *et al*., 1987). All these glycolipids were found antigenic and seemed to be promising species markers (Papa *et al*., 1987, Torgal-Garcia *et al*., 1988).

It is well known that *M. bovis* BCG contains a monoglycosylated 2-O-Me-α-L-Rhap phenolic glycolipid that is also found in *M. tuberculosis* strain Canetti (MacLennan *et al*., 1961, Daffe *et al*., 1988). Besides, this glycolipid and other phenolic glycolipids were identified in *M bovis* BCG (Puzo *et al*., 1989). These glycolipids could share common epitope with those of *M. tuberculosis* leading to false positive enzyme-linked immunosorbent assay tests when *M. tuberculosis* phenolic glycolipid was used for the screening of tuberculous patients. Also, by analogy to the immunological properties of the *M. leprae* it was assumed that presence of one of these phenolic glycolipids could be involved stimulation of the T suppressor cells, and confusing result observed in the protection against *M. tuberculosis* by *M. bovis* BCG (Mustafa *et al*., 1986). Thus isolation and purification of other minor phenolic glycolipids are key steps for further study of their activity on T-cells.

Earlier, only high-performance liquid chromatography (HPLC) has been reported to separate phenolic glycolipids from *M. bovis* BCG extract. By this method, phenolic glycolipids were separated by open column and reversed phase high performance liquid chromatography (RP-HPLC) using methanol-chloroform mixture as mobile phase. Analyses have been carried out by UV detector at 275nm (Puzo *et al*., 1989). Moreover, this report was confined to purify major phenolic glycolipids in *M. bovis* BCG by HPLC.

The main objective of this work was to investigate the performance of a simple and rapid method for the simultaneous separation of the phenolic glycolipids using

**Corresponding author:* Tel: +98 261 6102999; Fax: +98 261 6102900; e-mail: salimimona@pasteur.ac.ir, salimi_mona@yahoo.com

RP-HPLC. This analysis was provided a good resolution of the phenolic glycolipids, and allowed to observe minor glycolipids present in the crude extract of *M. bovis* BCG.

EXPERIMENTAL

Materials and chemicals

M. bovis BCG 1173P[2] was obtained from Pasteur Institute, Iran. The strain was grown on sauton's medium at 37°C and incubated for 28 days. Methanol and acetonitrile were of HPLC grade (Merck, Germany). Other purchased chemicals were of analytical grade (Merck, Germany). Double distilled water was prepared from a Maxima Purelab ultra-pure water purification system (ELGA Labwater, UK). The Mycoside B (a generous gift from Dr. Martin Gilleron of the Centre de Recherche de Biochimie et de Gttnttique Cellulaires du Centre National de la Recherche Scientifique, Toulouse, France) was used as standard.

Preparation of sample

M. bovis BCG cells (250g) upon hot extraction with CH_3OH-$CHCl_3$ (1:2; v/v) at 60°C for one day yield lipid extract (0.88g). Crude extract (0.88g) was dissolved in chloroform and then applied to an open column (14x 2.5cm) of silicic acid for separation (Fournie *et al*., 1987).

Column chromatography procedure

The column (14x2.5cm) was filled with silicic acid (Kieselgel G60, 230-400 mesh, Merck, Darmstadt, Germany). Then the column was irrigated with 500mL each of 25, 50, 75 and 100%(v/v) $CHCl_3$ in petroleum ether and followed by a gradient elution, 400mL each of 1, 2, 4, 8 and 10% CH_3OH in $CHCl_3$. All the steps of separation were checked by thin layer chromatography.

TLC analysis

Thin layer chromatography (TLC) was conducted with commercial silica gel plates (Kieselgel 60 F_{254} Merck, Darmstadt, Germany), developed in $CHCl_3$-CH_3OH (9:1; v/v), sprayed with 0.1% orcinol in 40% H_2SO_4 and heated at 111°C for 5 min. The glycolipids were shown with a brown-purple color.

Sep-Pak purification

To remove the interferences before HPLC, partial purification of the phenolic glycolipids was carried out by passage of the samples through Sep-Pack cartridge columns. Various columns for solid-phase extraction (C18 & Silica, Waters Associates, MA, USA) were tested concerning the characteristic of phenolic glycolipids and eluted by a gradient solvent of methanol in water (1→100%) and each step was monitored by TLC. The extraction procedure was repeated three times in order to increase the recoveries of the compounds.

HPLC analysis

A HPLC instrument (Wellchrom, Knauer, Germany) equipped with 4 channel K-1001 pumps, an auto sampler (Model Triathlon), a UV absorbance detector (model K-2600) and ChromGate software for acquisition and evaluating the data was used for the separation of phenolic glycolipids. A stainless steel analytical column Nucleosil 100 RP8 (125×4.0 mm i.d., 5µm, Knauer, Germany) connected to a Nucleosil 100 RP8 guard column was used. Solvents were filtered before use by a Milipore filtration unit (Millipore, USA). A constant flow rate of 0.8 mL/min was used during analysis. An optimum mobile phase composition was achieved by using different compositions of methanol-water and acetonitrile-water. The final composition was optimized as acetonitrile-water 0:100 followed by a gradient to 80:20 over 37 min. The other conditions were room temperature and detector wavelength at 275nm.

RESULTS AND DISCUSSION

Extraction and purification of phenolic glycolipids from M. bovis

The dried methanol-chloroform extract of *M. bovis* was treated with acetone which contained the phenolic glycolipids along with other minor glycolipid derivatives. From this mixture, mostly mycoside B was clearly identified on silicic acid TLC. However, the difficulty in detecting minor glycolipids was amplified by the overlapping of lipid components on TLC. In order to detect more polar unknown glycolipids, the acetone-soluble mixture was fractionated by one successive step, instead of two steps (Puzo *et al*., 1989), by silica-gel chromatography. In this step, the column was irrigated with petroleum ether to eliminate most of the apolar compounds with R_f higher than that of mycoside B (R_f of mycoside B as a standard is 0.7 in chloroform-methanol; 9:1 v/v) (Puzo *et al*., 1989). Then the column was mainly eluted with chloroform and 1, 2, 4, 8, 10% methanol in chloroform. Upon staining with 0.1% orcinol in sulfuric acid, one major spot with R_f: 0.7 similar to mycoside B as standard, and the other minor spots with R_f less than mycoside B were observed whose brown-purple color indicated characteristics of the glycolipids. These fractions were also re-purified by Sep-Pak cartridge (C18 & silica) with 1-100% methanol-water and pooled the eluates (fig. 1). Finally, the separation of eluate was accomplished by HPLC equipped with a 5-µm nucleosil (C8) analytical column.

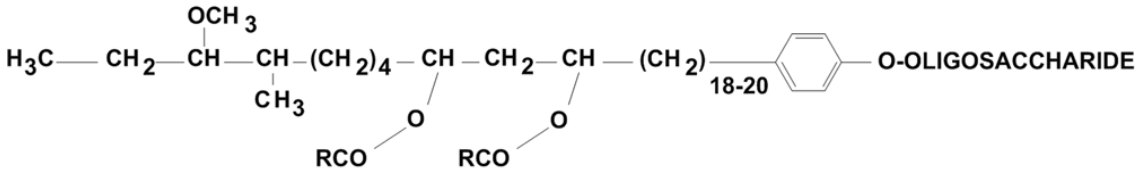
Optimization of solvent system of HPLC

Successful separation by HPLC largely depends on appropriate selection of column and a suitable solvent system which provide an optimum resolution of peaks for target compounds. The chemical structures of the phenolic glycolipids are similar to some degree (Figure 2) (Vercellone *et al*., 1992) but their solubility profiles are

different. This makes it difficult to detect them from a mixture by single method of HPLC. In the lipid extracts of *M. bovis*, a major phenolic glycolipid (mycoside B) was detected by TLC previously. The R_f values of PGLs which obtained from *M. bovis* in this study were compared with R_f value of standard mycoside B. Using TLC, the plates were examined for the spots development. Two of the phenolic glycolipid fractions, chloroform and 1% methanol, showed identical R_f value to mycoside B on TLC and other fractions with similar R_f comparative to phenolic glycolipids which identified previously (Puzo *et al*., 1989). Although the results of previous studies showed that TLC only can be a reliable alternative method for identification of phenolic glycolipids of *M. bovis* (Dandapat *et al*., 1999), we demonstrated a simple and new HPLC method for simultaneous detection and separation of PGLs. Three solvent system and two analytical columns were tested for RP-HPLC. All glycolipids were showed identical UV absorption spectra characteristic of an aromatic group at 275nm (Puzo *et al*., 1989), therefore peaks at this wave-length could be confirmed as phenolic glycolipids.

In these three solvent systems; methanol-water 0:100 to 70:30 during 30 min and 0:100 to 80:20 over 37 min; acetonitrile-water 0:100 to 80:20 in 37 min, the peak resolution of the last solvent system was high and the retention time was rather short. The result indicated that the peak resolution of the solvent system composed of methanol-water 0:100 to 70:30 over 30 min using C4 as an analytical column for chloroform and 2, 4% methanol fractions were poor (fig. 3) and then replaced it by C8 column. HPLC analysis of mycoside B as standard and chloroform or 1% methanol fraction was separated by C8 reversed-phase. It was found that with the same method but changing of different column exhibited long but identical retention time which approved our previous TLC data.

Fig. 1: Thin layer chromatography of the chloroform phenolic glycolipid fraction from *M. bovis* BCG. (1) The purified chloroform fraction by Sep-Pak cartridge (C18). (2) The mixture of purified and partially purified of chloroform fraction. (3) The partially purified chloroform fraction before Sep-Pak cartridge. The thin layer plate was run in $CHCl_3/CH_3OH$ (9:1) and sprayed with 0.1% orcinol in 40% H_2SO_4 then heated at 111°C for 5 min.



Fatty acid structure	Phenolglycol structure	Carbohydrate structure	Phenolic glycolipids from
			M. bovis BCG
Mycocerosic C_{26}, C_{27}, C_{29}, C_{30}	Phenolphthiocerol	2-*O*-Me-α-∟-Rha*p*	PheGl B
Mycocerosic C_{16}, C_{18}, C_{24}, C_{26}	Phenolphthiodolone	2-*O*-Me-α-∟-Rha*p*	PheGl B-1
Mycocerosic C_{26}, C_{27}, C_{29}	Phenolphthiodolone (major homolog)	α-∟-Rha*p*	PheGl B-2
Mycocerosic C_{26}, C_{27}, C_{29}	Phenolphthiocerol	α-∟-Rha*p*-(1→3)- 2-*O*-Me-α-∟-Rha*p*	PheGl B-3
			M.kansasii
Mycocerosic C_{29}, C_{30}, C_{32}	Phenolphthiocerol	2,6-dideoxy-4-*O*-Me- α-D-arabino-hex*p*-(1→3)-4-*O*-Ac-2-*O*-Me- α-∟-Fuc*p*-(1→3)-2-*O*-Me-α-∟-Rha*p*-(1→3)-2,4-di-*O*-Me- α-∟-Rha*p*	PheGl K-1
			M. marinum
Mycocerosic C_{24}, C_{27}, C_{30}	Phenolphthiocerol	3-*O*-Me-α-∟-Rha*p*	PheGl M

Fig. 2: Molecular structures of the phenolic glycolipids of Mycobacteria.

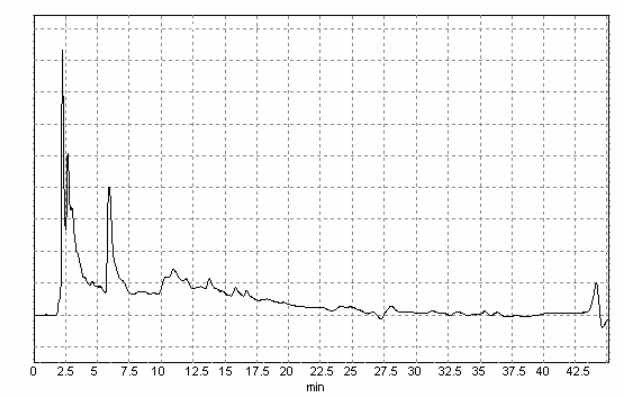


Fig. 3: HPLC-UV chromatogram of 4% methanol fraction of PGLs with C4 analytical column at a flow rate of 0.8 mL/min. The mobile phase was a gradient methanol-water from 0:100 to 70:30 over 30 min.

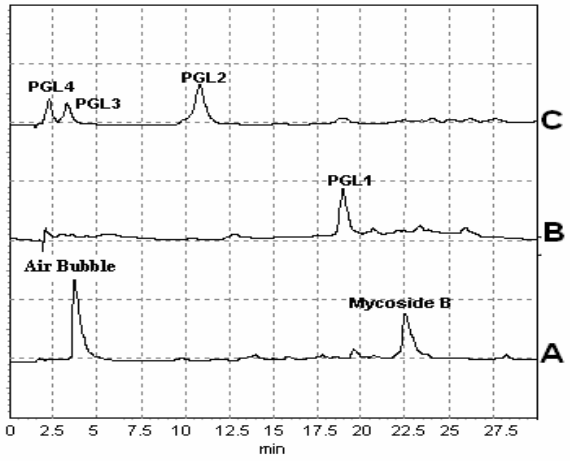


Fig. 4: HPLC-UV chromatogram of (A) chloroform (B) 2% methanol (C) 4% methanol fractions of PGLs with C8 analytical column at a flow rate of 0.8 mL/min. The mobile phase was a gradient from 0:100 to 80:20 of methanol in water over 37 min.

Therefore, we modified the solvent system from 0:100 methanol-water by a gradient to 80:20 over 37min showed better result (fig. 4). In order to decrease retention, further modification was performed by adding acetonitrile in different volume ratio. The retention time of few of the peaks were decreased and peak resolution increased. It was observed that acetonitrile-water from 0:100 to 80:20 over 37 min was produced the best separation than other solvent systems (fig. 5).

Our study indicated that the proposed method was simple and provided rapid separation of PGLs in *M. bovis*. A good resolution was obtained by using C8 column in RP-HPLC. A few new peaks were observed (fig. 5) which were absent in fig. 3. These preliminary results suggest that if phenolic glycolipids are needed to be purified by HPLC method, it may be advisable to purify it by RP-HPLC method on C8 column with gradient elution system.

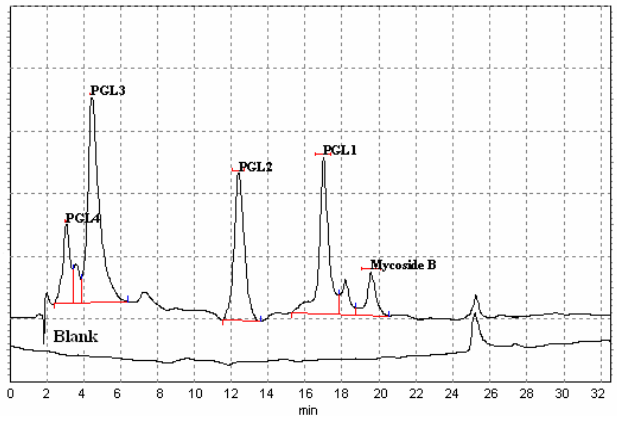


Fig. 5: Reversed-phase HPLC chromatogram of chloroform, 2, 4 and 8% methanol fractions of phenolic glycolipids. A C8 analytical column was used. The mobile phase was a linear gradient from 0:100 to 80:20 of acetonitrile in water over 37 min at a flow rate of 0.8 mL/min.

CONCLUSION

The previous study clearly demonstrated the complexity of the phenolic glycolipid fraction extracted from *M. bovis* BCG. To fractionate this mixture, a simple, rapid and nondestructive method based on gradient reversed-phase HPLC was successfully developed. The separation method described here might be applicable to the possible fractionation of a crude phenolic glycolipid mixture from other mycobacteria with similar structures. We established a simultaneous separation method for phenolic glycolipids (PGLs) of *M. bovis* by RP-HPLC. Although Puzo et al. (Puzo *et al*., 1989) indicated a separation method based on reversed-phase (RP) and normal-phase (NP) of HPLC, where the main eluent, chloroform, was incompatible with RP-columns. Besides, this analytical technique is very simple and efficient to provide a good resolution only by reversed-phase method which is very simple but time-consuming. Thus, to increase sensitivity in the aim of achieving a good resolution of the peaks, this newly developed method will be suitable for separation and purification of minor glycolipids present in *M. bovis* extract.

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