Waters column

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Mycolic Acids Using Nova-Pak C_{18}

Interest in how mycolic acids can be isolated and analyzed from biological matrices is increasing. For this reason, the need for a clearer understanding of what mycolic acids are and how they can be extracted from cells becomes increasingly important. The following is a definition of mycolic acids as described in the Merck Index, 11th edition:

Mycolic Acid: Is the term used to describe a class of compounds first obtained from a human strain of *Mycobacterium tuberculosis* and studied in an impure form called "unsaponifiable wax". The structure of mycolic acids varies by families and species. Three principal categories are known: (1) *corynomycolic acids* ranging from C_{28} to C_{40}, found mostly in Corynebacteria; (2) *nocardic acids*, also called nocardomycolic acids, ranging from C_{40} to C_{60}, produced by strains of *Nocardia*; and (3) mycobacterial mycolic acids ranging from C_{60} to C_{90}.

The following two articles discuss the isolation and analysis of mycolic acids in terms of public health and biotechnology perspectives using Nova-Pak® C_{18} columns.

Identification of Mycobacterium *Species*

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Tuberculosis caused by *Mycobacterium tuberculosis* is an increasingly important public health problem[1]. Several of the other *Mycobacterium* species such as *M. avium complex*, are also important causes of significant diseases. There have been several outbreaks of multidrug-resistant tuberculosis[2,3] in the United States. Multidrug-resistant tuberculosis refers to disease caused by *M. tuberculosis* isolates resistant to two or more of the commonly used antibiotics.

The genus *Mycobacterium* is characterized by slow-growing, "acid fast" bacilli believed to be gram positive. "Acid fastness"

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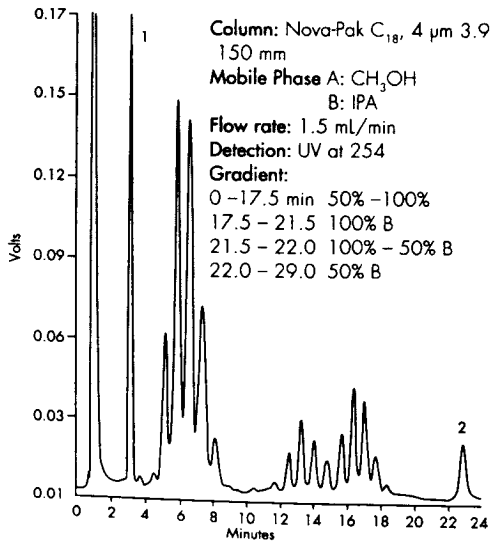
Figure 1: Optimized Separation of Mycolic Acids Using Nova-Pak C_{18} and Methanol/Isopropanol



Recent Developments in the Analysis of Mycolic Acids: Using HPLC Replacement of Methylene Chloride with IPA

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Ribi ImmunoChem Research, Inc.

Introduction

Mycolic acids (C_{70}-C_{90} α branched, ß-hydroxy fatty acids), a major part of mycobacterial cell walls, are a unique component of many mycobacterial species. For this reason, there has been great interest in using mycolic acid analysis to aid in the classification of mycobacteria. HPLC (Figure 1) has

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been utilized to rapidly separate mycolic acids, and the elution profile generated was diagnostic for several species [1, 2]. Ribi ImmunoChem Research, Inc. has developed high and low molecular weight internal standards for use in these analyses. At Ribi, the unique immunomodulating properties of mycobacterial cell walls are being exploited in the development of several pharmaceuticals. In order to identify the mycobacteria used in-house and to measure their mycolic acid content, certain modifications to the existing HPLC method were necessary.

Experimental

Isolation of the cell wall skeleton (CWS) from *Mycobacterium phlei* provided the source of mycolic acids. Samples were saponified, extracted, and derivatized with the UV chromophore p-bromophenacylbromide, as described previously [1]. In addition, the fluorescent reagent 4-bromomethyl-6,7-dimethoxycoumarin was also tested (data not shown). Six different internal standard compounds of increasing molecular weight and branching complexity (available from Ribi) were also used in these experiments (data not shown).

The chromatographic system was from Millipore, and included two Waters 510 pumps, 700 WISP, TCM, and either a 440 UV (254 nm) or a 470 fluorescence (Ex 345 nm, Em 425 nm) detector. The system was controlled via a SIM by Maxima (v 3.3).

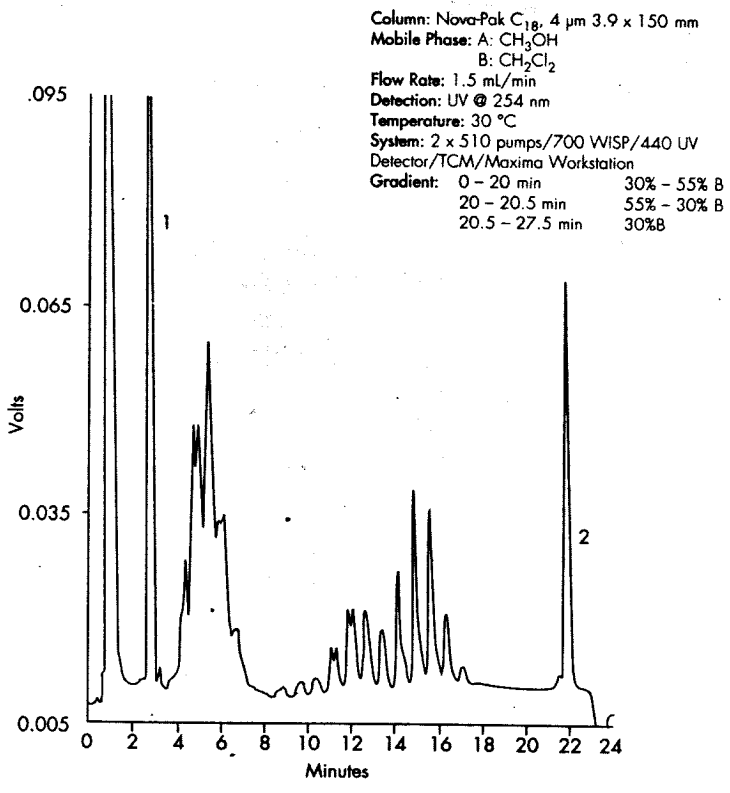
Results and Discussion

M. phlei CWS was chosen as a test sample because of its complex mycolic acid mixture, which tests the resolution limits of the HPLC assay. This species exhibits the early and late eluting groups of mycolic acids when analyzed by HPLC (e.g., 4 to 8 min and 11 to 17 min. respectively, Figure 1). Note that all of the figures show Ribi low (peak 1) and high (peak 2) MW internal standards, which can be used to generate relative retention times (RRT) for the mycolic acid peaks during the process of species classification. A Waters stainless steel Nova-Pak C_{18} 3.9 x 150 mm column (preceded by a Guard-Pak) and a Beckman Ultrasphere XL ODS 4.6x70 column (which was used in the published methods, [1, 2]) were tested. Methanol (MeOH), isopropanol (IPA) and methylene chloride ($MeCl_2$) were tested as eluents.

Each column was initially tested with MeOH/$MeCl_2$ gradients. The goal was to keep the total run time (including re-equilibration) to less than 30 min, so that an autosampler-equipped HPLC could be programmed to run at least 50 samples per day. With this constraint in mind, the chromatography of *M. phlei* mycolic acids was optimized at 30°C on the Nova-Pak column (Figure 4). The same sample analysis was also optimized at 30°C on the Beckman ODS column (Figure 5). Chromatographic resolution was very similar between the two columns under these conditions.

In light of the health hazards and solvent disposal costs associated with $MeCl_2$, IPA was tested in place of it. Runs of several IPA gradients revealed very different and rather striking results in comparison to those obtained with the use of $MeCl_2$. Figures 1 and 6 show optimized separations at 30°C on the Nova-Pak and Beckman

Figure 4: Mycolic acid profile derived from *M. phlei* using Nova-Pak C_{18}, 4µm and methylene chloride



columns, respectively. Compared to the use of $MeCl_2$, it is clear that the Nova-Pak C_{18} column exhibits superior performance to the Beckman ODS when IPA is used as the strong solvent.

One disadvantage of IPA is its high viscosity which leads to higher column backpressure. In order to overcome this problem analyses were run on the Nova-Pak column at 50°C, which lowered column backpressure by about 20% and shortened the sample analysis time by about 10%. To date, over 200 runs have been made on a single Nova-Pak C_{18} 3.9 x 150 mm column at 50 °C with no significant loss in resolution or increase in back-pressure.

With the series of Ribi standards the molar absorptivities of the analytes did not change with their carbon number or branching complexity. Thus, the method can be semi-quantitative if one computes generic response factors for the Ribi standards. The sensitivity of the assay was increased 10 - 20 fold by using the coumarin reagent; however, fluorescence was quenched by increased chain length so the method cannot be quantitative when this derivative is used (data not shown).

Conclusions

The method is safer to perform if IPA is used in place of $MeCl_2$, and it saves in disposal costs associated with chlorinated solvents. Ruggedness can be increased by heating the column above ambient temperature, and by using early and late eluting Ribi internal standards to enhance relative retention time (RRT) calculations. The method can be semiquantitative if the UV reagent, bromophenacylbromide derivative, is used. Sensitivity may be increased by using a fluorescent reagent.

References:

1. Butler, W.R., K.C. Jost, Jr., and J.O. Kilburn. *J. Clinical Microbiology*. 29(11)2468-2472. 1991.

2. Ramos, L.S. *American Biotechnology Lab*. 10(8)27. 32. 1992.

For ordering information, see page 29. For more information on Waters Nova-Pak columns and packings, please check box 4 on reply card.

Figure 5: Mycolic Acid Profile Using Beckman Ultrasphere and Methanol/Methylene Chloride

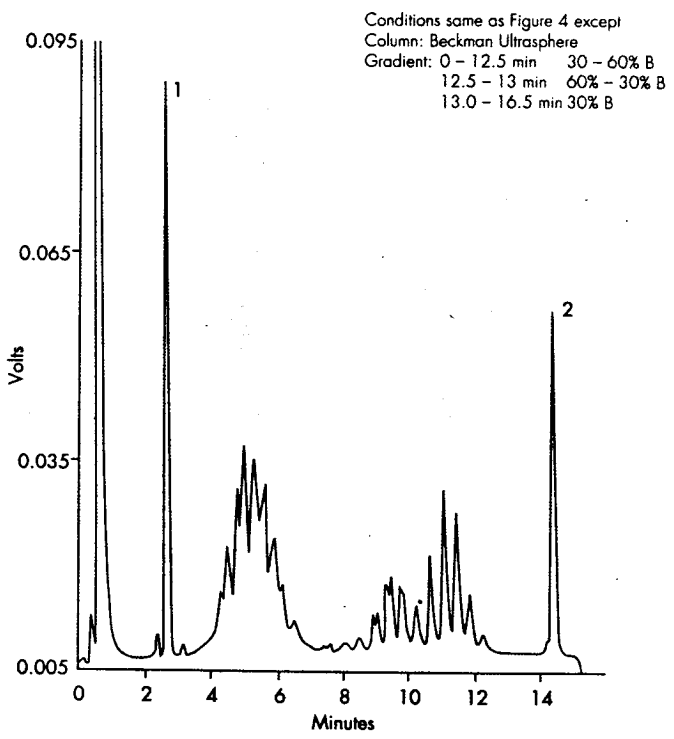


Figure 6: Mycolic Acid Profile Using Beckman Ultrasphere and Methanol/Isopropanol