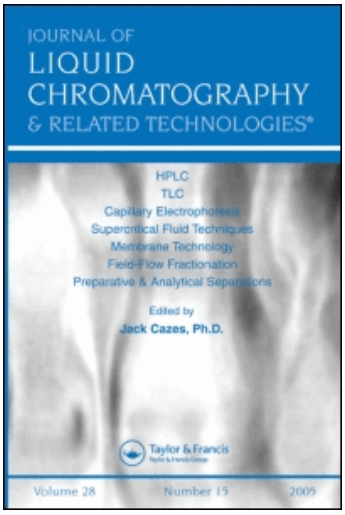

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DETERMINATION OF NEUTRAL SUGARS IN MYCOBACTERIAL CELL WALLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high-performance liquid chromatography method for the determination of neutral sugars in mycobacterial cell walls was developed. Cell wall samples were hydrolysed in either 1 N H_2SO_4 for 5 h at 100±2°C or 4 N TFA for 4 h at 100±2°C. The hydrolysed samples were applied to an ion moderated partition column using water as the eluent, and the column effluent was monitored by refractive index detection. Using acid treated neutral sugar standards the minimum calibration curve r^2 value was 0.996, the coefficient of variation of multiple determinations averaged 5.2% and the detection limit for each analyte was 0.2 μg.

INTRODUCTION

Mycobacterial cell walls have been shown to exhibit unique immunomodulating and anti-tumor properties, which are being exploited in the development of human pharmaceuticals [1]. The cell walls of mycobacteria

are composed of three types of polymers: peptidoglycan, cell wall associated proteins, and arabinogalactan (or arabinomannan) substituted with mycolic acids. The measurement of cell wall neutral sugars is necessary in structure/activity studies and bulk drug quantification during pharmaceutical development.

The neutral sugars in mycobacterial cell wall preparations have been measured by gas chromatography (GC), after pre-column derivatization of the sugars to their corresponding alditol acetates [2,3].

An comprehensive literature review revealed no high-performance liquid chromatography (HPLC) protocols for the analysis of mycobacterial cell wall neutral sugars. Consequently, a relatively simple HPLC technique utilizing refractive index (RI) detection was developed, which did not require pre-column derivatization of the reducing sugars.

MATERIALS AND METHODS

All of the chemicals used were of the highest available commercial purity. Arabinose (ARA), galactose (GAL), glucose (GLC) and trifluoroacetic acid (TFA) were from Sigma (St.Louis, MO, USA). *Mycobacterium phlei* and *M. fortuitum* cell wall skeleton (CWS) preparations were from Ribi ImmunoChem Research, Inc. (Hamilton, MT, USA). HPLC grade water was supplied by a Modulab type I water system from Continental (San Antonio, TX, USA).

Samples were hydrolyzed with 4 N TFA or 1 N H_2SO_4 (4.00 mg CWS/ml acid) in vacuum sealed glass tubes at 100±2°C. Working standards consisting of ARA, GAL and GLC (each at 0.500 mg/ml) in 4 N TFA or 1 N H_2SO_4 were similarly prepared. After TFA hydrolysis, standard and sample aliquots were desiccated, then re-suspended in equal volumes of HPLC water. After H_2SO_4 hydrolysis, standard and sample aliquots were neutralized with 0.5 N NaOH.

The HPLC (except for the column) was from Waters (Milford, MA, USA), and consisted of: a 700 autoinjector (200 μl sample loop); a 510 pump; a temperature control module (operated at 85°C); a 410 RI detector (40°C internal); computer control (via a system interface module) by Maxima (ver. 3.3). The column (Aminex HPX-87P, with a matching guard column) was from Bio-Rad (Richmond, CA, USA). For the analysis of H_2SO_4 treated standards and samples, a Bio-Rad deashing column was also used. The eluent for all of the analyses was degassed HPLC water, delivered isocratically at 0.5 ml/min.

RESULTS AND DISCUSSION

Based on previous analyses of bacterial polysaccharides, TFA and sulfuric acids were chosen for sample hydrolysis [2-4]. Experiments were conducted to determine the optimal hydrolysis conditions for neutral sugar release from the CWS preparations. It was found that hydrolysis in 1 N

H_2SO_4 for 5 h at 100±2°C, or in 4 N TFA for 4 h at 100±2°C resulted in optimal sugar release (data not shown). A typical chromatogram of TFA hydrolysed *M. phlei* CWS (50 μl injection) is shown in Figure 1.

The calibration curves for GLC, GAL and ARA in the working standards, whether H_2SO_4 or TFA treated, were consistently linear (minimum r^2 of 0.996) over the range of 1 to 20 μg per injection for each analyte (Table 1). In an additional test, linearity was demonstrated to at least 100 μg per

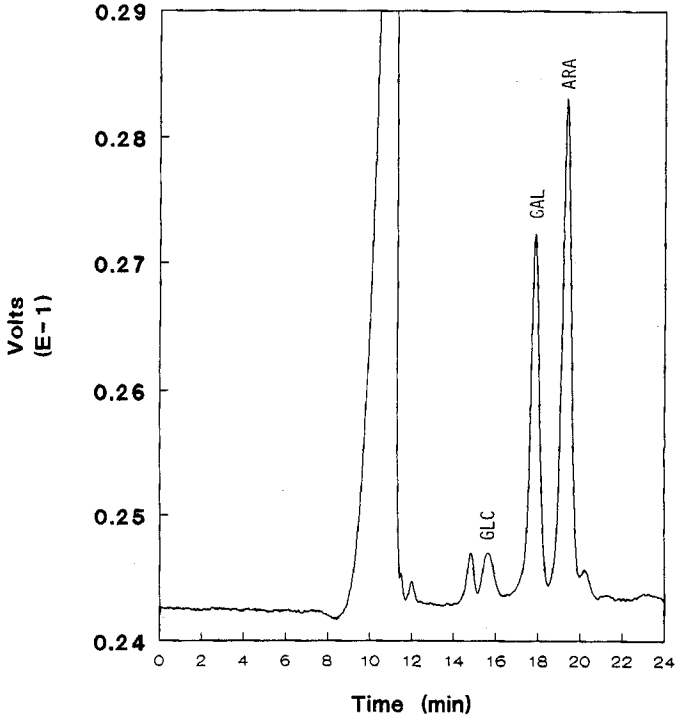

FIGURE 1. A typical chromatogram of TFA hydrolysed *M. phlei* CWS (50 μl injection).

TABLE 1

The standard curve linear regression data for the 1 N H_2SO_4 (5 h at 100±2°C) and 4 N TFA (4 h at 100±2°C) treated working standards is shown below. Standard curves were generated by making multiple analyses of each working standard, which represented 1, 2.5, 5, 10, 15 and 20 μg injections of each neutral sugar.

Analyte	Acid Trt	Slope	y-intercept	r^2
GLC	H_2SO_4	6232	-1264	0.998
GLC	TFA	5761	-1248	0.998
GAL	H_2SO_4	6079	-862	0.998
GAL	TFA	5325	-1167	0.996
ARA	H_2SO_4	5885	-620	0.999
ARA	TFA	5223	-1663	0.997

injection for each neutral sugar. The detection limit for GLC, GAL and ARA was determined to be 0.2 μg, corresponding to a signal-to-noise ratio of 2.

Precision, as measured by the coefficient of variation, averaged 5.2% and ranged from 0.6 to 14.3% (Table 2). The accuracy of the method was assessed through several standard addition experiments, by adding GLC, GAL and ARA to CWS preparations prior to acid hydrolysis. Recovery of standards after H_2SO_4 hydrolysis averaged 91%, while recovery of GLC, GAL and ARA after hydrolysis in TFA averaged 103% (Table 3).

Hydrolysis with H_2SO_4 resulted in lower neutral sugar values, especially for ARA, in comparison to TFA treatment (Table 2). Thus, the ARA/GAL ratios were also lower for H_2SO_4 treated samples (Table 2). The

TABLE 2

The neutral sugars in *M. fortuitum* and *M phlei* CWS preparations were determined after hydrolysis in 1 N H_2SO_4 for 5 h at 100±2°C, or hydrolysis in 4 N TFA for 4 h at 100±2°C. The average μg of each neutral sugar (± one standard deviation, four determinations) per miligram of CWS are shown.

CWS Prep	Acid Trt	GLC	GAL	ARA	ARA/GAL
M fort.	H_2SO_4	4 ± 0.2	137 ± 4	126 ± 5	0.91
M fort.	H_2SO_4	9 ± 1	96 ± 3	118 ± 5	1.23
M phlei	TFA	7 ± 1	148 ± 6	158 ± 15	1.06
M phlei	TFA	18 ± 0.1	101 ± 2	144 ± 2	1.42

TABLE 3

The percent recoveries of standards added to *M. fortuitum* and *M. phlei* CWS preparations were determined for both the 1 N H_2SO_4 (100±2°C for 5 h) and 4 N TFA (100±2°C for 4 h) hydrolysis treatments. The average percent recoveries (± one standard deviation, four determinations) for each neutral sugar are shown.

CWS Prep	Acid Trt	GLC	GAL	ARA
M phlei	H_2SO_4	95 ± 4	90 ± 7	87 ± 8
M phlei	TFA	98 ± 2	109 ± 8	109 ± 8
M fort.	TFA	98 ± 3	100 ± 4	102 ± 6

lower sugar values found after H_2SO_4 hydrolysis correlated with the lower overall recovery of standards added to CWS before H_2SO_4 hydrolysis (Table 3).

Previous GC determinations of the ARA/GAL ratio of several mycobacterial species, after 1 N H_2SO_4 hydrolysis for 5 h at 100°C, ranged from 2.33 to 2.76 [2]. In a more recent paper, the ratio for *M. tuberculosis*, after hydrolysis in 2 M TFA for 1 h at 121°C, was found to be 1.38 by GC analysis [3]. This latter value compares more favorably to the ratios reported in this paper (Table 2).

The low levels of GLC measured by the HPLC method (Table 2) were probably derived from glycogen which may have been trapped in the cell wall during CWS preparation. Additionally, the CWS preparations were checked for mannose (MAN) content with this method, by adding MAN (Sigma) to the working standards. Based on previous data [2,3] no MAN was expected to be present in CWS from *M. fortuitum* or *M. phlei*, and none was detected.

For the analysis of mycobacterial CWS preparations with the HPLC method, hydrolysis in 4 N TFA for 4 h at 100±2°C is recommended for two reasons. First, standard recoveries were higher in comparison to 1 N H_2SO_4 treatment (Table 3). Second, and ARA/GAL ratios in TFA treated samples (Table 2) more closely matched recent published data [3].

The HPLC method described herein should be widely applicable to the analysis of bacterial cell wall neutral sugars, and it is simpler to perform than the GC technique which has been described elsewhere [2-3].

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