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Analysis of mycolic acids by high-performance liquid chromatography and fluorimetric detection Implications for the identification of mycobacteria in clinical samples

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Abstract

Mycolic acids from *Mycobacterium phlei* and *M. bovis* cell wall skeletons (CWSs) were analyzed by HPLC. After saponifying lyophilized CWSs in methanolic KOH, the mycolic acids were quantitatively extracted into chloroform. Aliquots of the CWS mycolic acid extracts were then derivatized prior to HPLC analysis with a UV reagent, *p*-bromophenacylbromide (PBPB), and three fluorescent reagents, 4-bromomethyl-6,7-dimethoxycoumarin, 4-bromomethyl-7-acetoxycoumarin and 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one. A synthetic α-branched carboxylic acid was derivatized with the same reagents and used as an internal standard along with the mycolic acids. The derivatized samples were analyzed by reversed-phase HPLC on a Waters Novapak C_{18}, 4 μm particle size, 150 mm × 3.9 mm stainless-steel column. Two solvent systems were used: (1) methanol and methylene chloride with the column at 30°C, and (2) methanol and isopropanol with the column at 50°C. Detection sensitivity with the fluorescent reagents was 16–50 times greater than the sensitivity observed with PBPB-derivatized samples. Unique mycolic acid elution profiles for the two mycobacterial species could be achieved with each of the solvent systems and derivatization reagents tested. Thus, the HPLC analysis of pre-column derivatized mycolic acids was useful as a means of rapidly identifying mycobacterial species. Replacement of methylene chloride with isopropanol and PBPB with a fluorescent derivatizing reagent could increase the safety and sensitivity of the assay, and make it more useful for the clinical identification of mycobacterial infections.

1. Introduction

Mycobacterial infections, especially of the Mycobacterium tuberculosis group, represent an increasingly serious public health risk [1]. Improvement in the clinical diagnosis of these infections is dependent upon the availability of rapid diagnostic tests [2]. To date, the types of tests employed include: biochemical and physiological, genetic (DNA) probe assays, GC of cellular fatty acids and HPLC of mycolic acids (C_{60}–C_{90} α-branched, β-hydroxy fatty acids) [2–4].

HPLC analysis of the mycolic acids has emerged as the method of choice for the diagnosis of mycobacterial infections, due to its rapid and reproducible nature [2], and because the mycolic acid elution pattern observed for each mycobacterial species has generally been found to be unique [2,5–11].

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The HPLC analysis of the mycolic acids was performed as a three-phase process. First, the acids were derivatized pre-column with a UV chromophore, *p*-bromophenacylbromide (PBPB) [12]. Next, an HPLC separation was achieved in the reversed-phase mode using methanol and methylene chloride as eluents [8]. Finally, the chromatogram can be compared to a reference library and a species identification can be made [13].

Recently, some improvements over the existing technique have been reported. Detection sensitivity was dramatically improved by utilizing 4-bromomethyl-6,7-dimethoxycoumarin (BRDC) as a pre-column derivatizing reagent [14], followed by reversed-phase HPLC with fluorescence detection [15]. Chromatographic ruggedness has been increased by employing isopropanol (in place of methylene chloride) and temperature control during chromatography [16].

This paper discusses the combination of pre-column derivatization with fluorescent reagents, followed by temperature-controlled HPLC analysis using isopropanol in place of methylene chloride. In addition to BRDC, two other fluorescent reagents were tested: 4-bromomethyl-7-acetoxycoumarin (BRAC) [17] and 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one (BRMB) [18].

2. Experimental

2.1. Reagents

HPLC-grade acetonitrile (ACN), chloroform ($CHCl_3$), isopropanol (IPA), methanol (MeOH), methylene chloride ($MeCl_2$), and reagent-grade acetone, concentrated hydrochloric acid (HCl), anhydrous magnesium sulphate ($MgSO_4$), potassium hydrogencarbonate ($KHCO_3$), potassium hydroxide (KOH) were obtained from J.T. Baker (Phillipsburg, NJ, USA). BRDC and 18:crown:6 ether were obtained from Aldrich (Milwaukee, WI, USA). PBPB crown ether solution in acetonitrile was obtained from Pierce (Rockford, IL, USA). BRAC was obtained from Regis (Morton Grove, IL, USA). BRMB was obtained from TCI America (Portland, OR, USA). Mycobacterial cell wall skeleton (CWS) preparations and a proprietary α-branched carboxylic acid internal standard compound were obtained from Ribi ImmunoChem Research (Hamilton, MT, USA).

2.2. CWS saponification and quantitative extraction

CWS preparations from *Mycobacterium phlei* and *M. bovis* served as sources of mycolic acids. CWS samples were saponified in 25% (w/v) KOH in MeOH–water (1:1, v/v) (5 mg CWS per ml KOH solution) for 2.5 h at 102.5 ± 2.5°C. After heating, the samples were placed in an ice bath in a fume hood, and concentrated HCl (0.076 ml per ml KOH solution) was carefully added. The resulting mixtures were extracted three times with $CHCl_3$ (0.8 ml $CHCl_3$ per ml KOH solution). Centrifugation followed each extraction, and the lower (organic) layers were collected. The combined $CHCl_3$ extract was dried under a stream of nitrogen, then quantitatively transferred with $CHCl_3$ washes into a tared tube through a luer-lok syringe fitted with a Gelman (Ann Arbor, MI, USA) 0.2-μm Acrodisc filter. Finally, the solvent was removed under a stream of nitrogen, and the tube was reweighed to obtain a known mass of CWS mycolic acid extract.

2.3. Pre-column derivatization

For each derivatization reagent test, a sample consisted of the following: 0.400 mg of mycolic acid extract (from either mycobacterial species) plus 39.4 nmol of the Ribi internal standard compound.

Derivatization with PBPB was as described previously [6], except that the samples were heated for 30 min at 85 ± 2°C.

Coupling with BRDC was achieved in generally the same manner as previously described [15]. Briefly, 100 μl of 20 mg per ml $KHCO_3$ in MeOH–water (1:1, v/v) was added to each mycolic acid/internal standard sample, with subsequent drying under a stream of nitrogen. Next,

500 μl of chloroform, 175 μl of 2.5 mg/ml BRDC in ACN (dried over $MgSO_4$) and 25 μl of 10.6 mg/ml 18:crown:6 in acetone (dried over $MgSO_4$) were added. The samples were then heated for 30 min at 50 ± 2°C. Finally, the solvent was removed under a stream of nitrogen. Reaction with BRAC and BRMB was identical, except that 2.5 mg/ml BRAC in dried acetone and 2.3 mg/ml BRMB in dried ACN were used.

2.4. Chromatography

The chromatographic system was from Waters, and included two 510 pumps, a 700 WISP, a TCM (30 or 50°C) and either a 440 UV (254 nm) or a 470 fluorescence detector (16-μl flow cell). Fluorescence excitation (ex) and emission (em) wavelengths were as follows: BRDC, ex = 345 nm, em = 425 nm; BRAC ex = 315 nm, em = 400 nm; BRMB ex = 340 nm, em = 415 nm. The system was controlled, and data were collected and processed with Waters Millenium (version 2.0) software. A Waters stainless-steel Novapak C_{18} 150 × 3.9 mm column (preceded by a Guard-pak equipped with a C_{18} insert) was used.

Prior to HPLC analysis, samples were dissolved in $CHCl_3$–MeOH (2:1, v/v).

Depending on the reagent being tested, various linear solvent programs using MeOH and $MeCl_2$, a column temperature of 30°C and a flow-rate of 1.5 ml/min were run (data not shown). Similarly, separations were achieved by using IPA in place of $MeCl_2$, a column temperature of 50°C and a flow-rate of 1.5 ml/min (Table 1).

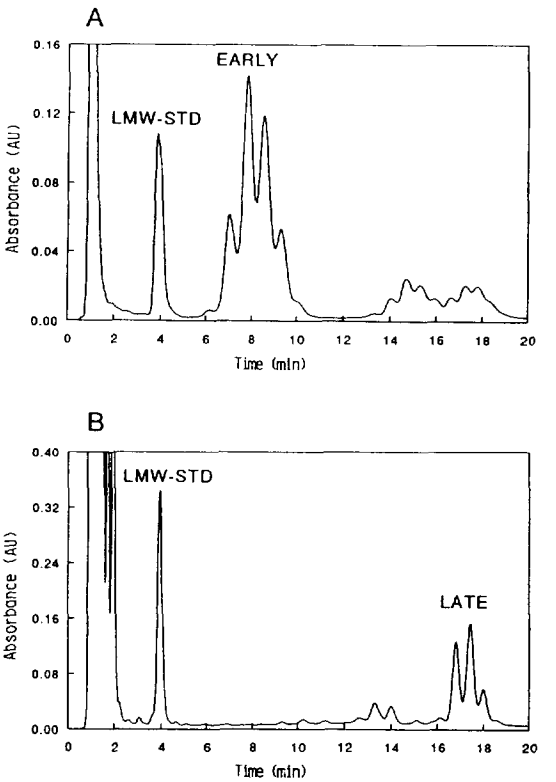

Fig. 1. Representative chromatograms of PBPB-derivatized mycolic acids from *M. phlei* (A) and *M. bovis* (B) separated with an MeOH–IPA solvent gradient (see Table 1).

3. Results and discussion

M. phlei and *M. bovis* CWS samples were used in these experiments for two reasons. First, extracts from these samples exhibited the early (*M. phlei*, see Fig. 1) and late (*M. bovis*, see Fig. 1) eluting mycolic acid groups during HPLC

Table 1
Percentages of MeOH and IPA which were used to achieve separations of mycolic acids derivatized with four reagents

Time (min)	PBPB		BRDC		BRAC		BRMB	
	MeOH	IPA	MeOH	IPA	MeOH	IPA	MeOH	IPA
0.0	60.0	40.0	80.0	20.0	85.0	15.0	72.5	27.5
18.0	6.0	94.0	10.0	90.0	10.0	90.0	10.0	90.0
18.5	60.0	40.0	80.0	20.0	85.0	15.0	72.5	27.5
25.5	60.0	40.0	80.0	20.0	85.0	15.0	72.5	27.5

analysis [16]. Thus, while only two species were tested, they yielded a complex pattern of mycolic acids which served to test the applicability of the HPLC method using elution with IPA and fluorescence detection. Second, sufficient quantities of these preparations were available for quantitative mycolic acid extraction, which was essential for the accurate determination of detection limits (discussed below).

In previous applications of the HPLC method, a "high-molecular-mass" internal standard compound (HMW-STD, Ribi ImmunoChem Research) has been used for the calculation of relative retention times (RRTs), which aid subsequent mycobacterial species identifications [2,7–11]. This compound was supplied as the PBPB ester, and has been the latest eluting peak of interest during the HPLC analysis [2,6–10].

In order to test a variety of derivatizing reagents, a lower-molecular-mass α-branched non-esterified carboxylic acid was used. This compound, identified as LMW-STD in Figs. 1–4, eluted prior to the mycolic acid peaks. Since it was available in a highly pure form, accurate determinations of the detection limits for each of the derivatizing reagents could be made (discussed below).

In general, literature protocols were followed for the preparation of the chromogenic and fluorogenic mycolic acid esters [12,14,17,18]. In order to achieve reproducible coupling of the mycolic acids and the internal standard with the fluorescent reagents, it was necessary to use $MgSO_4$-dried acetone and ACN (data not shown). The reagent concentrations, reaction times and temperatures used for sample derivatization were found to be optimal (data not shown).

Two gradient elution solvent systems were tested for the HPLC analysis of the esterified mycolic acids: MeOH and $MeCl_2$ (data not shown), MeOH and IPA (Table 1, Figs. 1–4). Two constraints were placed on the optimization of separation with the eluents tested. First, the total run time for the analysis, including injection delay and gradient plus column re-equilibration (1 min plus 25.5 min, respectively) was arbitrarily set at 26.5 min. This represented

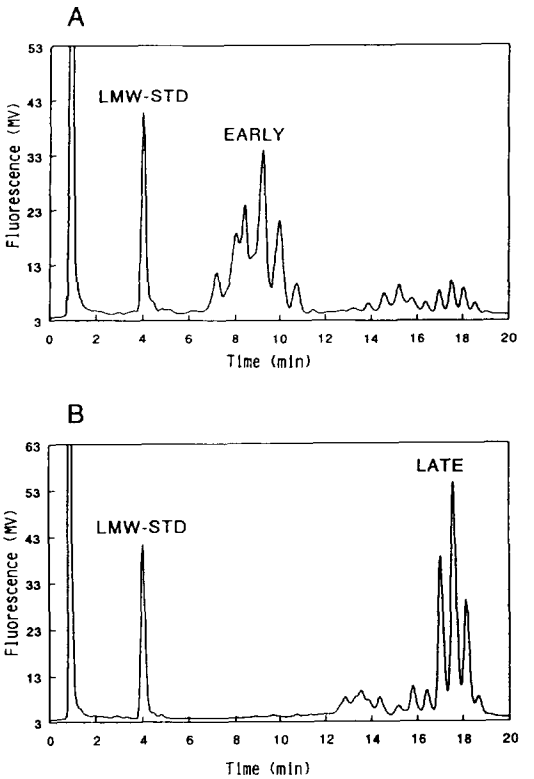

Fig. 2. Representative chromatograms of BRDC-derivatized mycolic acids from *M. phlei* (A) and *M. bovis* (B) separated with an MeOH–IPA solvent gradient (see Table 1).

a compromise between analytical speed and resolution. Second, the solvent gradient conditions were set so that the internal standard compound eluted at about 4 min for each of the reagents tested. This allowed for meaningful comparisons of the various reagents in different elution environments.

Detection limits were assessed by making HPLC analyses of serially diluted samples derivatized with PBPB and each of the fluorescent reagents. The detection limits for the internal standard are expressed as three times the average amplitude of the detector baseline noise, and ranged from 6.00 pmol for the UV reagent, PBPB to 0.12 to 0.24 pmol for the fluorescent reagents (Table 2). Detection limits for the mycolic acid extract represent the approximate minimum mass that was required for a positive species identification by the HPLC technique.

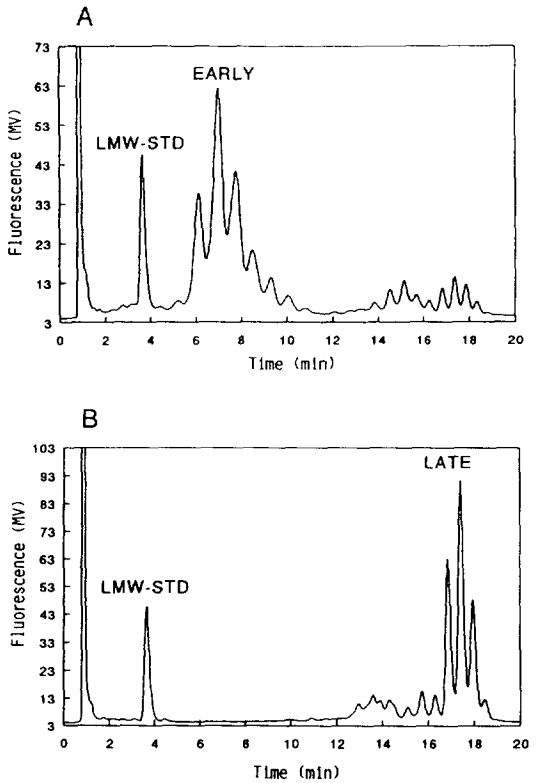

Fig. 3. Representative chromatograms of BRAC-derivatized mycolic acids from *M. phlei* (A) and *M. bovis* (B) separated with an MeOH–IPA solvent gradient (see Table 1).

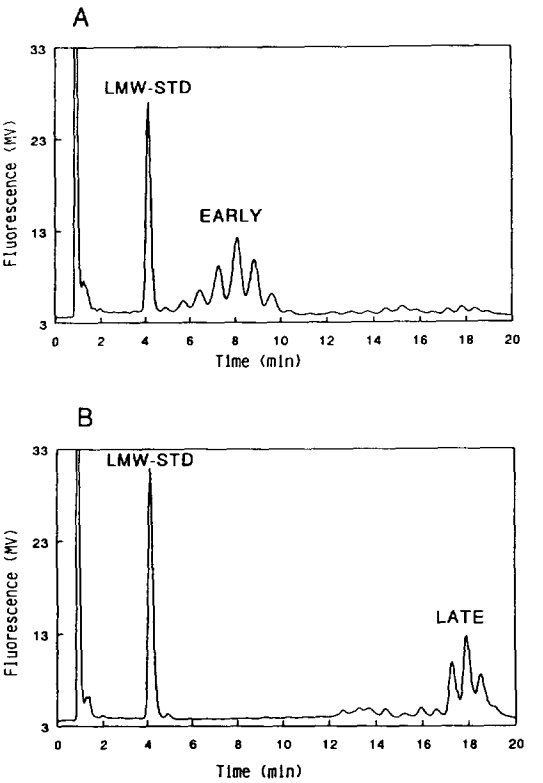

Fig. 4. Representative chromatograms of BRMB-derivatized mycolic acids from *M. phlei* (A) and *M. bovis* (B) separated with an MeOH–IPA solvent gradient (see Table 1).

These ranged from a high of 800 ng for PBPB to 30–50 ng for the fluorescent reagents (Table 2). Thus, depending on how detection limit was assessed (standard acid vs. acid extract), the fluorescent mycolic acid esters were 16–50-fold more sensitive than the PBPB esters. The results with BRDC were similar to those reported previously [15]. In light of the fact that detection limits with BRAC were enhanced about 10-fold further by employing a post-column reaction system [17], the potential exists for a 500-fold increase in sensitivity over the UV detection of PBPB esters.

While imposing the analysis and retention time constraints discussed previously, HPLC separations were optimized. Figs. 1–4 show representative chromatograms generated with MeOH–IPA for extracts from each species derivatized with PBPB and the three fluorophores. Chromatograms were similarly obtained by using MeOH–$MeCl_2$, but are not shown.

Several observations were made as a result of these analyses. The fluorescent adducts were more polar than the PBPB derivatives, the polarity of the derivatized esters was as follows: BRAC > BRDC > BRMB > PBPB. Peak resolu-

Table 2
Detection limits (MDL, corresponding to a signal-to-noise ratio of 3) for a branched-chain carboxylic acid internal standard compound, and for representative mycolic acid extracts

Reagent	MDL acid (pmol)	MDL extract (ng)
PBPB	6.00	800
BRDC	0.19	40
BRAC	0.12	30
BRMB	0.24	50

tion was as follows in MeOH–IPA gradients: BRDC > BRAC > BRMB > PBPB. There was a selectivity (peak resolution) change when MeOH–$MeCl_2$ gradients were used; peak resolution was as follows: BRDC > PBPB > BRMB > BRAC (data not shown).

The results point to several improvements in the ruggedness of the current HPLC technique [2,7–11]. One improvement would be to use temperature control (above ambient conditions) and IPA in place of $MeCl_2$, as previously noted [16]. Chromatography using MeOH–IPA gradients and column temperature control at 50°C resulted in improved retention time reproducibility (as compared to analyses where ambient temperatures fluctuated significantly) and an approximately 25% lower column backpressure. Also, IPA is safer to use than $MeCl_2$, and does not cavitate in the HPLC pump heads at high operating temperatures and elevations. By using the LMW-STD in conjunction with the HMW-STD, it may be possible to bracket the mycolic acids and generate more reproducible RRTs during the process of species identification.

Finally, these experiments indicate that the clinical culture time before mycobacterial sampling could be reduced if PBPB is replaced with a more sensitive fluorescent reagent (e.g., BRDC, BRAC, BRMB). Also, the altered chromatographic selectivities of the fluorescent mycolic acid esters (shown here and previously [15]) may be useful in the optimization of HPLC for clinical mycobacterial species identification.

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