The Separation of the Mycobactins from *Mycobacterium smegmatis* by using High-Pressure Liquid Chromatography

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The family of mycobactins from *Mycobacterium smegmatis* were resolved into seven fractions by high-pressure liquid chromatography. This separation was by virtue of the differences in length and character of the long acyl substituents as shown by g.l.c. of the methyl esters of the isolated fatty acids from the fractions. As t.l.c. could also resolve the individual mycobactin fractions, it too must rely on the same differences to effect separation. As the lengths of the acyl chains were modulated by the growth conditions, a specific range of acyl groups may not be needed for mycobactin to function. This technique provides a simple means of rapidly characterizing crude mycobactins from all mycobacteria.

Mycobactins are lipid-soluble iron-binding compounds isolated from all species of mycobacteria, except for a few species that depend on mycobactin as an essential growth factor (Snow, 1970). The iron-chelating properties of the mycobactins are due to the presence of two hydroxamate groups and a phenolic hydroxy group in juxtaposition to the N atom of an oxazoline ring. This chelating nucleus is made lipid-soluble by the attachment of a long acyl chain. Mycobactins show species differences in the nature of the substituents in the chelating nucleus, but when isolated from a single species show a common nucleus with acyl chains of differing lengths. Thus White & Snow (1969) showed that the acyl chains of mycobactin S (i.e. from *Mycobacterium smegmatis*) were derived from a mixture of the Δ^2 -*cis*-C₁₄, -C₁₆, -C₁₈ and -C₂₀ monoenoic fatty acids. This was accomplished by degradation of the mycobactin, isolation of the fatty acids and their subsequent vapour-phase chromatography as methyl esters.

In this paper we show that it is possible to fractionate the family of mycobactins from *M. smegmatis* by h.p.l.c. by virtue of the differences in lengths of the acyl substituents. As this can be done on crude mycobactin, it provides a simple aid for the characterization of these molecules.

Methods

Mycobactin

M. smegmatis N.C.I.B. 8548 was grown with shaking, unless stated otherwise, on iron-deficient medium (0.05 µg of Fe/ml) to promote synthesis of myco-

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

bactin as previously described (Ratledge & Hall, 1971). Mycobactin S was extracted and purified as its ferri complex as described by Ratledge & Snow (1974) for the purification of nocobactin. The ferri-mycobactin was finally passed through a column of Sephadex LH20, with ethanol as solvent. The final A₄₃₀ of 44.3 was a slight improvement over the previously recorded value of 42.8 (White & Snow, 1969).

High-pressure liquid chromatography (h.p.l.c.)

A Varian 8500 pumping system was connected via a stopped-flow injector to a column (50 mm × 2 mm) packed with Partisil Si-10-ODS. A Perkin-Elmer LC55 spectrophotometer coupled to a 0.5 mV recorder was used to monitor the eluate. Relative proportions of eluted components were estimated by triangulation of repeated chromatograms. The mobile phase was a mixture of methanol (analytical-reagent grade) and water in the ratio 4:1 (v/v), later changed to 10:3 (v/v) for better resolution of two components. The flow rate was 120 ml/h in both cases, under 7.6 MPa pressure.

Gas-liquid chromatography of fatty acids derived from mycobactin

Mycobactins, ferri form (about 2-10 mg), were refluxed with methanol (1 ml) and 6 M-HCl (1 ml) for 4 h. The solution, now colourless, was extracted with diethyl ether (2 × 3 ml). The ether was washed with water (3 ml), evaporated to 1 ml and then treated with 0.1 ml of diazomethane in ether (approx. 1 M; 15 min at 3°C). The methyl esters were then chromatographed by using a column (1.5 m × 5 mm) packed with 10% (w/w) diethylene

glycol succinate on 80–100-mesh Supelcoport (Chromatography Services Co., Wirral, Merseyside, U.K.) held isothermally at 175°C with N₂ as carrier gas. Identifications were made from appropriate standards of saturated and Δ^9 -unsaturated fatty acids; Δ^2 -*cis*-monoenoic acids were not identified as such, but were presumed to be so from their retention times and the previously published data of White & Snow (1969). Relative proportions of the eluted esters were calculated by triangulation of their peak areas.

Results

Reversed-phase h.p.l.c. analysis of purified ferri-mycobactins by using methanol/water (4:1, v/v) gave a six-component chromatograph (Fig. 1) when monitored at 450 nm, the visible absorption maximum for these compounds. This wavelength was used for all subsequent work, although the chromatographic profile was unchanged at the more sensitive wavelength of 217 nm. This clear separation into several components offers a possible means of characterizing mycobactin families.

A variety of other chromatographic conditions were studied, including mixtures of methanol/hexan-1-ol/water as mobile phase, but the relative retention volumes of the six components were little changed. Fraction 2 could be partly resolved into two components, 2A and 2B, under conditions in which fraction 6 was broadened beyond the limit of detection.

The reproducibility of the h.p.l.c. fractionation under a variety of conditions suggests a separation on the basis of molecular structure. The most likely basis for this differentiation of a mycobactin family is the length and degree of unsaturation of the acyl groups, and to substantiate this we attempted to identify the acyl group of the mycobactins corresponding to the six components. Repeated collections were made of each of the six components as they were eluted from the analytical column. Clean fractionation was not expected, owing to the broadness of the peaks (typical of reversed-phase liquid chromatography), particularly for the slowest running components, and to the difficulty of taking centre cuts for the fast-running components. The effectiveness of this attempt at a physical separation was tested by re-analysing each of the separated fractions (Table 1). As expected, the purity of collected fraction 1 was high, but the others were progressively more contaminated by the earlier components. The purity of fraction 6 was exceptionally bad, reflecting the long collection time (2–3 min) for the corresponding h.p.l.c. peak. The purity of this collected fraction would be improved by substantially shortening the retention time (and hence the band width), but only at the expense of losing the resolution between the

Fig. 1. Elution profile of mycobactin S on h.p.l.c. Conditions of operation are described in the Methods section; the solvent was methanol/water (4:1, v/v). Numbers on peaks correspond to six identifiable fractions. Peak 2 has a shoulder on its right side and is a mixture of two components.

earlier components. In the isolation of fraction 2, the proportion of fraction 2B was enhanced (from 1/2.7 to 1/1.36), presumably because of the loss of fraction 2A into fraction 1 on its collection.

Each of the six collected fractions (fraction 2 containing both components 2A and 2B) was analysed for its constituent fatty acids by g.l.c. of the methyl esters. Although over 20 acids were detected, many of these were present at very low concentrations. To ensure a reasonably reliable quantitative

Table 1. *H.p.l.c. of mycobactin and the various fractions isolated from h.p.l.c. of the original mycobactin (see Fig. 1)*
Conditions of operation were as given in the Methods section, with methanol/water (4:1, v/v).

Peak no. (see Fig. 1)	Relative percentage (w/w) of the six peaks in the elution profile of mycobactin						
	Original*	Fraction† 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6
1	6.6	94.8	4.8	3.0	12.7	13.3	39.1
2	46.7	5.2	95.2	4.3	8.1	6.7	27.2
3	31.2	0	0	82.4	8.4	3.1	11.2
4	7.1	0	0	10.3	70.1	1.7	4.8
5	7.1	0	0	0	0.7	73.7	3.1
6	1.3	0	0	0	0	1.5	14.6

* Average of six chromatograms.

† Fractions collected by repeated h.p.l.c. of original mycobactin.

Table 2. *Percentage distribution (by g.l.c. analysis) of fatty acid methyl esters from mycobactin and in collected h.p.l.c. fractions of mycobactin*

Many trace components were detected, but for reliable quantitative analysis components constituting less than 3% were ignored. Carbon number was assigned according to Woodford & Van Gent (1960). Under 'Possible identity', unspecified monoenoic acids were assumed to be with the double bond in the usual position (see Hung & Walker, 1970).

Possible h.p.l.c. peak	Carbon number	Possible identity	Original mycobactin	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6
1	10.0	C _{10:0}		10					
	10.3	Δ ² -cis-C _{10:1}	3	37	7	13	7	7	22
	12.0	C _{12:0}		8			4	4	
	12.6	C _{12:1}		4					
	14.0	C _{14:0}	21	17	11	8	8	7	5
2	15.6	C _{15:1}		5					
	16.0	C _{16:0}	17	13	35	4	12	16	19
	16.2	Δ ² -cis-C _{16:1}	26		34			4	6
	17.6	C _{17:1}	5		13				
3	18.2	Δ ² -cis-C _{18:1}	14	6		51	7	3	4
	18.6	C _{18:1}							7
	19.7					18	4		
4	18.0	C _{18:0}	14			7	56	4	5
	19.5	C _{19:1}						5	7
5	20.3	Δ ² -cis-C _{20:1}						45	7
	21.7							4	
6	20.0	C _{20:0}							16

analysis, g.l.c. peaks contributing 3% or less were ignored. The results of the g.l.c. analysis are given in Table 2 and show a close correspondence to the h.p.l.c. results after allowing for the contamination with other components. Peak 1 of the h.p.l.c. profile consists of some seven fatty acyl moieties ranging from C_{10:0} to C_{15:1}, whereas peak 2 consists of Δ²-cis-C_{16:1} (2A) and C_{16:0} with C_{17:1} (2B). Peak 3 is mainly Δ²-cis-C_{18:1}, with an unidentified acyl group with an equivalent carbon chain length of 19.7. Peaks 4, 5 and 6 are essentially one component each, respectively C_{18:0}, Δ²-cis-C_{20:1} and C_{20:0}.

Thus mycobactins were eluted by h.p.l.c. in order of their acyl-chain length, but with mycobactins

with unsaturated acyl chains emerging before saturated ones.

T.l.c. of the original mycobactin, and of six fractions produced by h.p.l.c., could separate the mycobactins (Table 3). Furthermore, fractions 1 and 2 were now each seen to be running as two spots. By comparisons with the other fractions, the faster-running spot of fraction 2 was probably component 2A (i.e. that with the unsaturated acid). The faster-running spot of fraction 1 was probably also a component with a Δ²-cis-unsaturated acid (i.e. the C_{10:1} acid; see Table 2), whereas the slower spot probably contained saturated acid(s) (C_{10:0}, C_{12:0} and/or C_{14:0}). As there was some streaking between

Table 3. *Thin-layer chromatography of mycobactins*
Ferrimycobactins, original material and fractions produced by h.p.l.c. were chromatographed on 250 μ m-thick layers of alumina G by using a double development of cyclohexane/ethyl acetate/methanol (50:50:1, by vol.).

Mycobactin	R_f * value	Streak
Original	0.62–0.95	
Fraction 1	0.61, 0.74	Two spots
Fraction 2	0.74, 0.91	Two spots
Fraction 3	0.98	
Fraction 4	0.79	
Fraction 5	1.00	
Fraction 6	0.86	

* Migration relative to fastest moving spot.

the two main spots it is probable that all three acids were being partially resolved. There had been no indication during h.p.l.c. that fraction 1 could be even partially resolved into components under the conditions used to detect all six fractions. Separation of mycobactins by t.l.c., of course, can only be accomplished, in this case, after fractionation by h.p.l.c. It would be no use with the original material, as this produced a long streak with no distinction of the individual mycobactins.

The efficacy of h.p.l.c. in separating mycobactins was demonstrated by showing that the same elution profile, as given by the purified material (Fig. 1), was obtained with crude, freshly isolated mycobactin. Hence the technique could be applied without recourse to the lengthy purification process that would be required for g.l.c. analysis. The technique was therefore used to examine the crude mycobactins from *M. smegmatis* grown under different conditions.

The mycobactins taken from 3-day-old cultures, instead of the usual 5 days, showed a slight change in the composition of the principal fractions (Table 4). Fractions 2B and 3 appeared to have undergone reciprocal changes, with the former increasing and the latter decreasing by the same amount. Far greater changes in the mycobactins occurred when either 1% (v/v) Tween 80, a common constituent of mycobacterial growth media, was included in the medium or when the cultures were grown without shaking (Table 4). With bacteria grown in the presence of Tween 80, a new peak (1A) was seen, indicating the presence of an acyl group less than ten carbon atoms. There was also considerable enhancement of peaks 1 and 2A, with peaks 3, 4 and 5 being considerably decreased. Thus the oleic acid of the Tween 80 [poly(oxyethylene sorbitan mono)oleate] was not being accommodated in the mycobactin, but was being degraded to short-chain fatty acids. Growth under stationary conditions produced a

Table 4. *H.p.l.c. of mycobactins isolated, but not purified, from M. smegmatis grown under various conditions*
Conditions of chromatography: methanol/water (10:3, v/v) at 120 ml/h and under 7.6 MPa (75 atm.) pressure. Total elution time was about 10 min. Results are averages of two duplicate chromatographs.

Condition of growth	Relative percentage (w/w) of mycobactin peaks						
	1A*	1	2A	2B	3	4	5
Control (5-day culture)	0	6	34	12	33	7	8
3-day culture	0	8	32	19	26	10	5
+1% (v/v) Tween 80	2	17	57	3	17	0	4
Stationary culture	0	7	54	2	30	2	5

* Fast-running peak eluted before the usual first fraction.

Table 5. *H.p.l.c. of mycobactins eluted from M. smegmatis with increasing concentrations of ethanol*

Air-dried iron-deficient cells were extracted with 55% (v/v) ethanol for 24 h, filtered and placed in the next higher concentration of ethanol. The process was repeated daily with increasing concentrations of ethanol. Results are averages of two duplicate chromatograms.

Ethanol concn.	Relative percentage (w/w) of mycobactin peaks					
	1	2A	2B	3	4	5
55% (v/v)	12	45	13	24	3	3
60% (v/v)	11	42	12	27	4	3
75% (v/v)	6	35	13	33	6	7
95% (v/v)	5	33	12	32	8	8

mycobactin having an enhanced component 2A with concomitant decreases in components of fractions 2B and 4, i.e. with less-saturated acyl groups.

The lengths of the acyl chain attached to the mycobactin are therefore changeable to some degree, which may mean that a range of acyl-chain lengths is not needed for mycobactin to fulfil its function.

The various mycobactins within *M. smegmatis* were not preferentially extracted to any marked degree when increasing concentrations of ethanol were used for the extraction (Table 5). Even with 55% (v/v) ethanol (the lowest concentration at which mycobactin will extract from *M. smegmatis*), mycobactins with all acyl-chain lengths were recovered. There was, however, enhancement of extraction of the shorter-chained mycobactins with the lowest ethanol concentration.

Discussion

The application of h.p.l.c. as a rapid means of examining mycobactins without their purification

has been demonstrated. Separation of the individual mycobactins is by virtue of their differing lengths of acyl chain. Thus a means of 'fingerprinting' mycobactins from various species now seems possible and h.p.l.c. may therefore be considered as complementary to t.l.c. in the examination of crude mycobactins. Any comparison of mycobactins taken from different species must take into account the possible influences of growth conditions on the nature of acyl chain, as these acyl groups are not inviolable.

Mycobactins, and nocobactins, which have been isolated from various species and reported as running as two or more discrete spots upon t.l.c. (Snow, 1970; Ratledge & Patel, 1976), may do so because of possessing acyl chains with widely disparate numbers of carbon atoms. Although one cannot estimate the length of the acyl chain by t.l.c.,

because of unknown contributions to mobility from the central nucleus, it may be possible to do so by using h.p.l.c.

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