Journal of Chromatography, 104 (1975) 460–464

CHROM. 7841

Note

Qualitative and quantitative thin-layer chromatography of mycolic acids in *Mycobacterium tuberculosis* var. *bovis*-BCG

Č. MICHALEC
Laboratory of Protein Metabolism, Faculty of General Medicine, Charles University, Prague (Czechoslovakia)
M. MÁRA
Institute of Hygiene and Epidemiology, PTM-BCG, Prague (Czechoslovakia)
and
D. ADAMCOVÁ
Laboratory of Special Medical Microbiology, Faculty of General Medicine, Charles University, Prague (Czechoslovakia)
(Received August 1st, 1974)

Mycolic acids are characteristic constituents in Mycobacteria, especially as they form the essential lipid part of biologically active substances (wax D, "cord factor", "PmKo factor") with important toxic and immunoadjuvant properties[1]. For this reason, the determination of the content of mycolic acids in whole lipids or individual lipid fractions, and the determination of their ratios with other fatty acids and with each other, are very important. On the other hand, the concentration of mycolic acids could be an indirect parameter of the content of these substances in individual strains of Mycobacteria and also a determining factor of their biological activity.

At present, the analytical methods for the determination of mycolic acids are very unsatisfactory. The most effective are the chromatographic techniques, *e.g.*, paper chromatography[2,3] and pyrolysis gas chromatography[4]. Thin-layer chromatography of methyl esters of mycolic acids was described by Winder and Collins[5].

As none of the above techniques solved the problem of the separation of individual types of mycolic acids in *Mycobacterium tuberculosis* var. *hominis* and *Mycobacterium tuberculosis* var. *bovis*-BCG, we have studied this question using thin-layer chromatography. The main aim of this study was to develop a simple and rapid screening method for the qualitative and quantitative determination of these substances in various strains of Mycobacteria, especially under different conditions of cultivation[6].

EXPERIMENTAL

Mycolic acids were isolated from *Mycobacterium tuberculosis* var. bovis-BCG (strain Prague 725) cultured on Sauton medium (asparagine was replaced with enzymatic casein hydrolyzate). Lipids were extracted for 48 h with 96% ethanol in

a Soxhlet apparatus. After hydrolysis with a 2.5% (w/v) solution of potassium hydroxide in methanol–benzene (1:1, v/v) for 6 h and extraction with diethyl ether, mycolic acids were precipitated with two volumes of 96% ethanol according to the procedure of Winder and Collins[5]. This extraction was repeated three times.

The purified samples were dissolved in diethyl ether or diethyl ether–chloroform (1:1) and chromatographed on Silufol sheets (4 × 9 cm) six times to the front of the solvent in *n*-hexane–diethyl ether (85:15). After each run, the solvent mixture was renewed.

Detection

The spots were detected by means of the following reagents.

(a) Acid fuchsin–uranyl nitrate reagent[7]. After drying, the chromatogram was sprayed lightly with a 0.02% solution of acid fuchsin in 0.01 *N* hydrochloric acid containing 0.2% of uranyl nitrate and then dipped for 10 min in the same reagent. The excess of the reagent was washed off with 0.2% of uranyl nitrate in 0.01 *N* hydrochloric acid. Mycolic acids gave red spots on a colourless background.

(b) Bromothymol blue–sodium hydroxide reagent[8]. The chromatogram was sprayed with a solution of 0.04% bromothymol blue in 0.01 *N* sodium hydroxide solution and then immersed for 2 min in the same reagent. After drying overnight, the chromatogram was dipped for 2 min in 0.25 *N* sodium hydroxide solution and then dried in air. Mycolic acids gave blue spots on an almost colourless background.

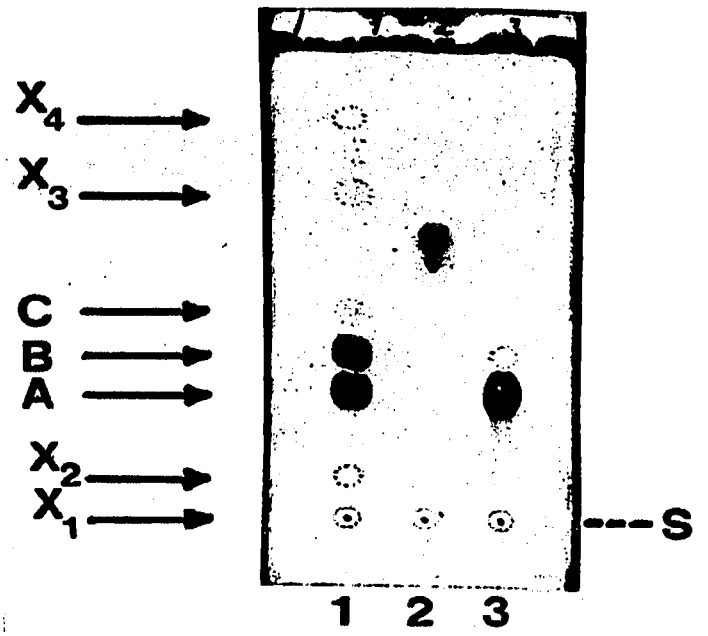

Fig. 1. Separation of mycolic acids from *Mycobacterium tuberculosis* var. *bovis*-BCG (strain Prague 725). Adsorbent: Silufol. Solvent system: *n*-hexane–diethyl ether (85:15). Detection: bromothymol blue–sodium hydroxide solution. 1 = Mycolic acids from *Mycobacterium tuberculosis* var. *bovis*-BCG; 2 = mixture of C_{16}–C_{30} fatty acids; 3 = mycolic acid (highly purified fraction A); A, B and C = mycolic acids; X_1 and X_2 = unidentified substances; X_3 and X_4 = higher fatty acids.

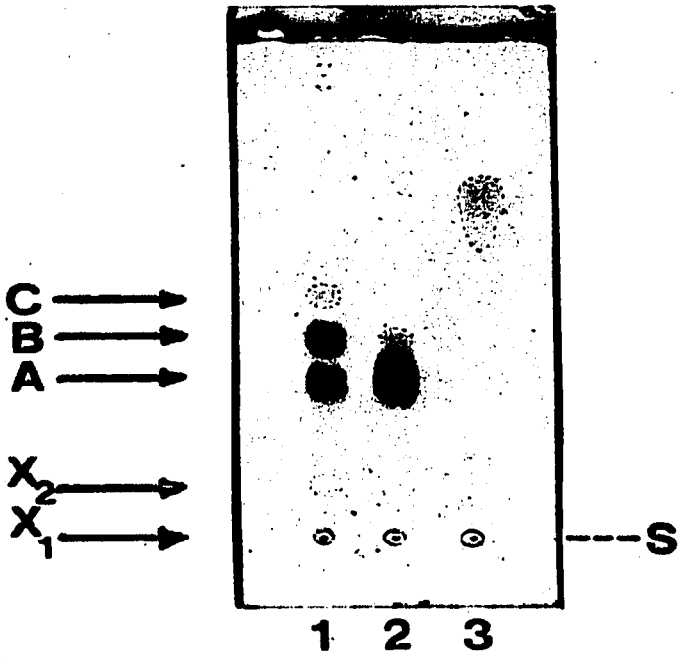

Fig. 2. Separation of mycolic acids from *Mycobacterium tuberculosis* var. *bovis*-BCG (strain Prague 725). Adsorbent: Silufol. Solvent system: *n*-hexane–diethyl ether (85:15). Detection: acid fuchsin–uranyl nitrate. 1 = Mycolic acids from *Mycobacterium tuberculosis* var. *bovis*-BCG; 2 = mycolic acid (highly purified fraction A); 3 = mixture of C_{16}–C_{30} fatty acids; A, B and C = mycolic acids; X_1 and X_2 = unidentified substances.

(c) Copper(II) acetate–rubeanic acid[9]. The chromatogram was sprayed lightly with a solution of 0.8% copper(II) acetate containing 0.05% of sodium acetate and then immersed in the same solution for 10 min. After washing in running water (3–4 h) and dipping in a saturated solution of rubeanic acid in water, the chromatogram was washed for 15 min with water. Mycolic acids gave grey-green spots.

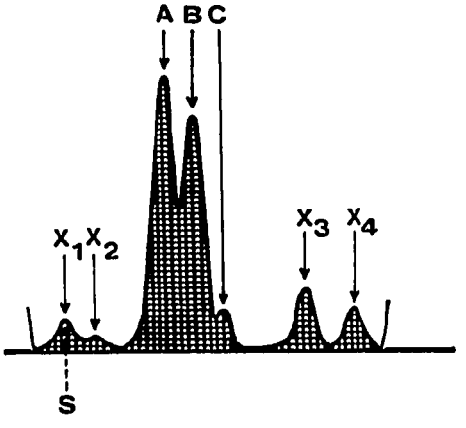

Fig. 3. Densitometry of mycolic acids. Experimental conditions and specifications as in Fig. 1.

TABLE I

R_F VALUES OF MYCOLIC ACIDS ISOLATED FROM *Mycobacterium tuberculosis*, VAR. *bovis*-BCG (STRAIN PRAGUE 725)

Fraction	R_F*
A	1.21
B	1.00
C	0.77
Higher fatty acids	1.65

* Relative to the mobility of fraction B (R_F = 1.00). The values are averages of 20 determinations.

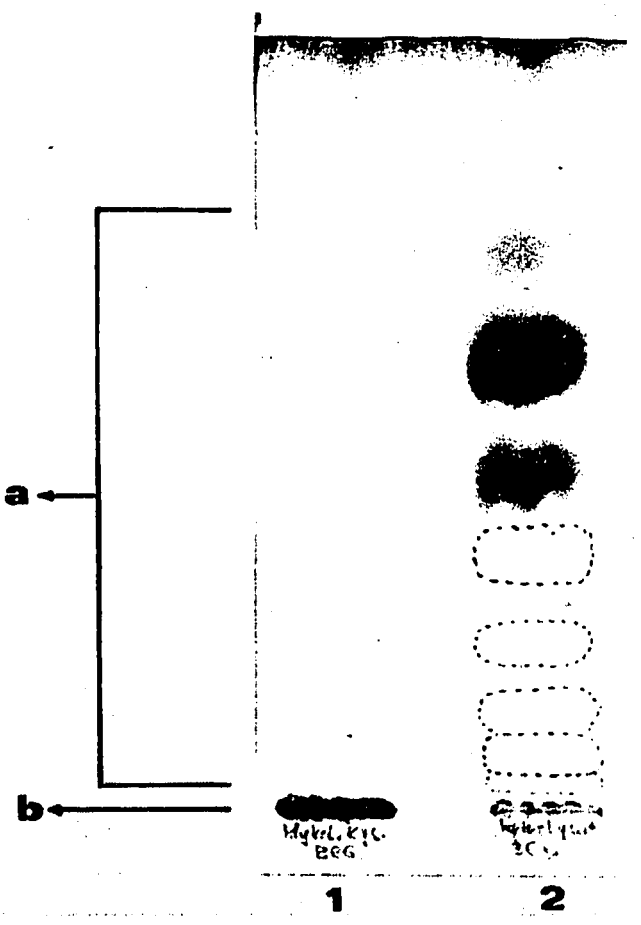

Fig. 4. Paper chromatography of mycolic acids. Paper: Whatman No. 3 impregnated with 5% paraffin oil. Solvent system: 90% acetic acid. Detection: copper(II) acetate–rubeanic acid. 1 = Mycolic acids from *Mycobacterium tuberculosis* var. *bovis*-BCG; 2 = hydrolyzate of whole lipids from *Mycobacterium tuberculosis* var. *bovis*-BCG; b = mycolic acids; a = higher fatty acids.

The chromatograms were scanned using an ERI-10 densitometer (Carl Zeiss Jena, Jena, G.D.R.) in reflected light.

RESULTS AND DISCUSSION

As can be seen from Figs. 1 and 2, the spectrum of mycolic acids isolated from *Mycobacterium tuberculosis* var. *bovis*-BCG consists of three main fractions A, B, and C. These findings are in close agreement with the results of some other workers, *e.g.*, Asselineau[10] and Winder and Collins[5]. Asselineau[10], using the same material, described three mycolic acids, α_1, α_2 and β. Winder and Collins[5] observed the same composition. A densitogram obtained using the same conditions as in Fig. 1 is shown in Fig. 3. R_F values are given in Table I.

The spots with lower mobilities (marked as X_1 and X_2 on the chromatograms) remain unidentified. On the other hand, the spots with higher R_F values represent higher fatty acids (about C_{30} or lower, X_3 and X_4). The identity of all three fractions of mycolic acids was verified by paper chromatography, in which all of these substances formed only one spot on the starting line (Fig. 4).

The application of this method for tracing the mycolic acid spectrum in various strains of Mycobacteria will be described elsewhere[6].

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