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HPLC Analysis of Mycolic Acids in Evaluation of Drug Susceptibility of *Mycobacterium tuberculosis* Strains – Comparison with Conventional Methods

RENATA WALKIEWICZ, RYSZARDA CHAZAN, HANNA GRUBEK-JAWORSKA*

Department of Internal Medicine, Pneumology and Allergology, Medical University of Warsaw, Warsaw, Poland

The commonly used phenotypic tests of *M.tuberculosis* drug susceptibility evaluate drug effects on quantity of colony forming units (CFU) on solid media or CO_2 secretion/O_2 consumption by cultures in liquid media. These tests are not precise enough and need a long time for the growth of the bacilli, so new more accurate and rapid methods are highly needed. The aim of this study was to determine the utility of quantitative HPLC analysis of mycolic acids for drug susceptibility of *M.tuberculosis*. In 119/120 (99.16%) of the performed tests the HPLC methods showed excellent agreement with the conventional phenotypic tests. The quantitative HPLC analysis of mycolic acids is a fairly exact indicator of tubercle bacilli growth and may be used as a quick and reliable measure of their drug sensitivity.

K e y w o r d s: HPLC, mycolic acids, drug susceptibility, *M. tuberculosis*

1. Introduction

Tuberculosis is a major cause of illness and death worldwide [1], and trend of the recent significant increase in the initial and multi-drug resistance (MDR) of *M.tuberculosis* isolates has been documented in subsequent WHO reports [2–5]. In this situation development of the potent faster-acting anti-mycobacterial compounds and introduction of the more precise and quicker tests of drug susceptibility is urgently needed.

The phenotypic tests of *M. tuberculosis* drug susceptibility evaluate the drug effects on quantity of colony forming units (CFU) on solid media or CO_2 secretion/O_2

* Correspondence to: Hanna Grubek-Jaworska, Department of Internal Medicine, Pneumology and Allergology, Medical University of Warsaw, ul. S. Banacha 1a, 02-097 Warsaw, Poland, e-mail: hgrubek@wum.edu.pl
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consumption by cultures in liquid media. These tests are commonly used in routine examination of the drug susceptibility of mycobacterial isolates in clinical laboratory [6, 7] as well as in the studies of anti-mycobacterial activity of new synthesised compounds [8, 9].

Concerning the clinical isolates, there are numerous reports on use of the molecular techniques to detect mutations related to the drug resistance [10–13], and some molecular tests are approved for routine diagnostics of the clinical isolates [14, 15] although not all resistance-related genes for the different anti-tuberculosis drugs have been found. Then it is commonly accepted that phenotypic tests which assess inhibition of *M.tbc* growth in the presence of drugs are still more reliable, recommended and commonly used in evaluation of the drug susceptibility in clinical practice [6, 7].

In laboratory evaluation of anti-mycobacterial activity of new synthesized compounds for understandable reasons only phenotype testing is in use. Because the extremely slow growth of bacilli, especially on solid media, the drug susceptibility tests require several weeks, so introduction of rapid and accurate techniques are desirable.

Quantitation of total mycolic acids by HPLC in liquid cultures of tubercle bacilli (with/without drug) enables accurate susceptibility testing within a few hours. Mycolic acids are branched high-molecular-weight β-hydroxy fatty acids with a long alkyl chain at the α-position. They form part of the mycobacterial cell wall, up to 30% of the cellular dry weight. Currently qualitative HPLC analysis of p-bromophenacyl esters of mycolic acids is recommended as a rapid and accurate method of identification of mycobacteria to species level on the basis of differences in HPLC elution profiles [16–19]. Recently quantitative HPLC analysis of mycolic acids is proposed in the phenotyping tests of *M.tuberculosis* drug susceptibility.

The aim of this study was to compare the results of *M.tbc* drug resistance assay by quantitative HPLC analysis of mycolic acids with those obtained by conventional tests – the proportion method on solid Loewenstein-Jensen (L-J) medium and detection of oxygen consumption in Mycobacteria Growth Indicator Tube on liquid medium (MGIT system). We investigated the resistance of 30 *M.tuberculosis* clinical isolates to the first-line anti-tuberculous drugs: isoniazid (INH), rifampicin (RMP), ethambutol (EMB) and the secondary drug – streptomycin (SM).

2. Materials and Methods

2.1. *M.tbc* Strains

A total of 30 strains of *M.tuberculosis* isolated from TB patients at the Department of Internal Medicine, Pneumology and Allergology of The Medical University of Warsaw and at the Otwock Center for Treatment of Lung Diseases were analyzed.

2.2. Drug Susceptibility Testing

a) analysis of mycolic acids

Mycolic acid analyses were carried out by HPLC following a modified method described previously, recommended by the Center for Disease Control and Prevention for typing to species level of clinical isolates of mycobacteria and used in our laboratory in routine practice [17,18]. Briefly: the procedure included saponification of thermally inactivated bacilli from a 5-ml culture in 7H9 Middelbrook broth (Becton, Dickinson & Co., Spark, MD, USA) with oleic acid-albumin-dextrose-catalase (OADC) with 2 ml of methanolic KOH solution (w/v 20% KOH in v/v 50% methanol), acidification with 1 ml 6 M HCl, and extraction into 2 ml of chloroform. The next steps were derivatization of 1ml of extracted mycolic acids to p-bromophenacyl esters and extraction into 1 ml of chloroform. Then, 0.8 ml of the extract was evaporated and solubilized in 40 µl of dichloromethane containing internal standards – low and high molecular weight mycolic acids (RIBI Immunochem Research Inc., Hamilton, MT, USA). Finally, 5 µl of the bromophenacyl derivatives was injected into a System Gold high performance liquid chromatograph (Beckman Instruments, Inc., San Ramon, CA, USA). Separation was performed on a Symmetry® C18 chromatographic column (Waters Corp., Milford, MA, USA) in methanol-dichloromethane gradient with detected spectrophotometric detection at 260 nm. The total area under mycolic acids peaks (TAMA) was determined automatically (System Gold software). The average error of TAMA analysis was +/– 9.5%.

b) proportion method

The test was performed according to a standard procedure [20]. The critical proportion for resistance was taken as 1% of mycobacterial population. If the number of colonies on medium with a drug was higher than 1% of that on control medium, the isolate was considered as resistant. To determine the 1% proportion of resistance, the inoculum seeded in the control L-J slant was 100-fold less than those seeded on the slants with drugs. The reading of growth was taken after 28 days. The qualitative evaluation was expressed as susceptible (S) or resistant (R).

c) MGIT™ AST SIRE system (Mycobacteria Growth Indicator Tube for Antimycobacterial Susceptibility Testing; Becton Dickinson & Co).

The system uses fluorescence technology and appropriate detectors are placed in tubes with 7H9 medium enriched with OADC. Their fluorescence is quenched by oxygen dissolved in the medium. Oxygen consumption by the growing mycobacteria permits the detectors to fluorescence under UV exposition. For quantitative evaluation of the fluorescence, the manual method using Bactec®Micro MGIT was applied [21].

d) drug concentrations

Concentrations of the drugs used in our study are presented in Table1 [22].

Table 1. Concentrations of drugs used in study

Antibiotic conc. (mcg/ml)	Method of analysis		
	HPLC	Proportion	MGIT
INH	0.05 0.10* 0.20	0.20*	0.10*
RMP	0.50 1.00* 2.00	40.0*	1.00*
EMB	1.75 3.50* 7.00	2.0*	3.50*
SM	0.40 0.80* 1.60	4.0*	0.80*

* Critical concentration.

3. Results

Figure 1 shows a typical HPLC elution pattern of bromophenacyl esters of mycolic acids extracted from 5-day culture of *M.tuberculosis* in 7H9 liquid medium. The part of profile indicated with broken lines was evaluated automatically as the total

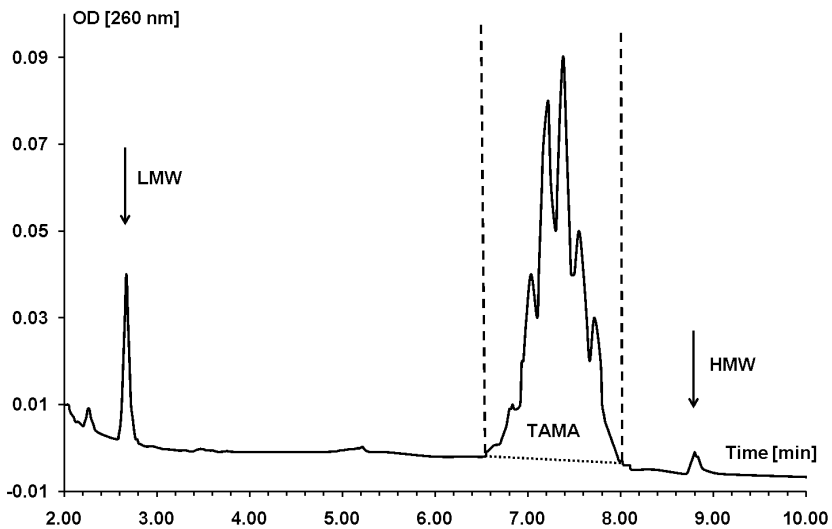

Fig. 1. HPLC elution profile of mycolic acids *M.tuberculosis*. The total area under mycolic acids peaks (TAMA) was determined automatically (Beckman System Gold). ↓ – low (LMW) and high (HMW) molecular weight standards of mycolic acids. The average error of TAMA analysis was +/– 9.5%

area under mycolic acids peaks (TAMA). In the preliminary experiments the kinetics of *M.tuberculosis* growth evaluated by TAMA in relation to inoculum size was determined (Fig. 2). The option of 5-day cultures with inoculum <0.5 according to McFarland was selected for the further experiments. Figure 3 compares the typical

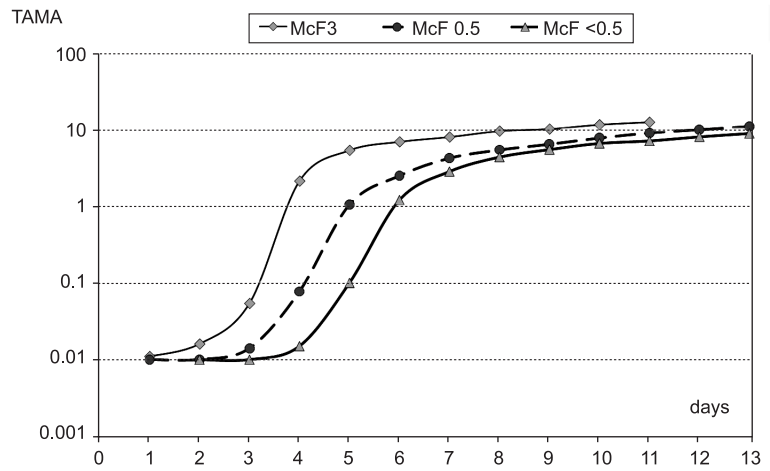

Fig. 2. *M.tuberculosis* growth in drug-free medium in relation to size of inoculum

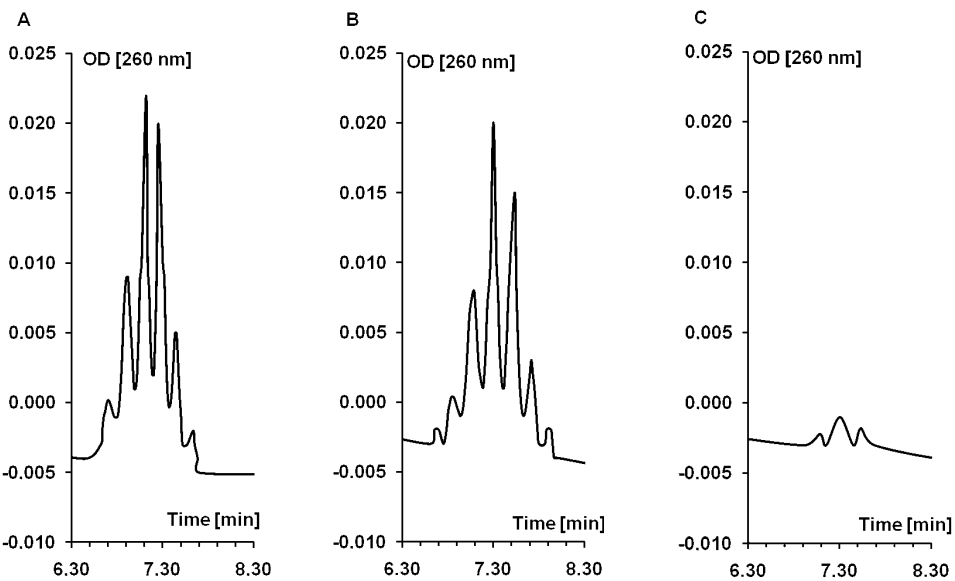

Fig. 3. Typical HPLC profiles of mycolic acids of drug-resistant and drug-sensitive bacilli grown in drug free medium and in the presence of drug: A – control culture, B – cultures with drug – resistant strains, C – cultures with drug – susceptible strains

HPLC profiles and the ranges of TAMA values of bromophenacyl esters of mycolic acids extracted from drug-resistant and drug-sensitive strains in 5-day cultures in 7H9 medium with a drug. The MAI value i.e. TAMA with drug / TAMA of control culture, was calculated for each strain grown in presence of INH, RMP, EMB or SM used at increasing concentrations. Typical graphs illustrating the MAI values for SM-sensitive (n = 22) and SM-resistant (n = 8) strains as a function of SM concentration of the drug are shown in Fig. 4. Similar relations were observed with INH, RMP, EMB (Table 2). Figure 5 illustrates the ranges of MAI values determined for

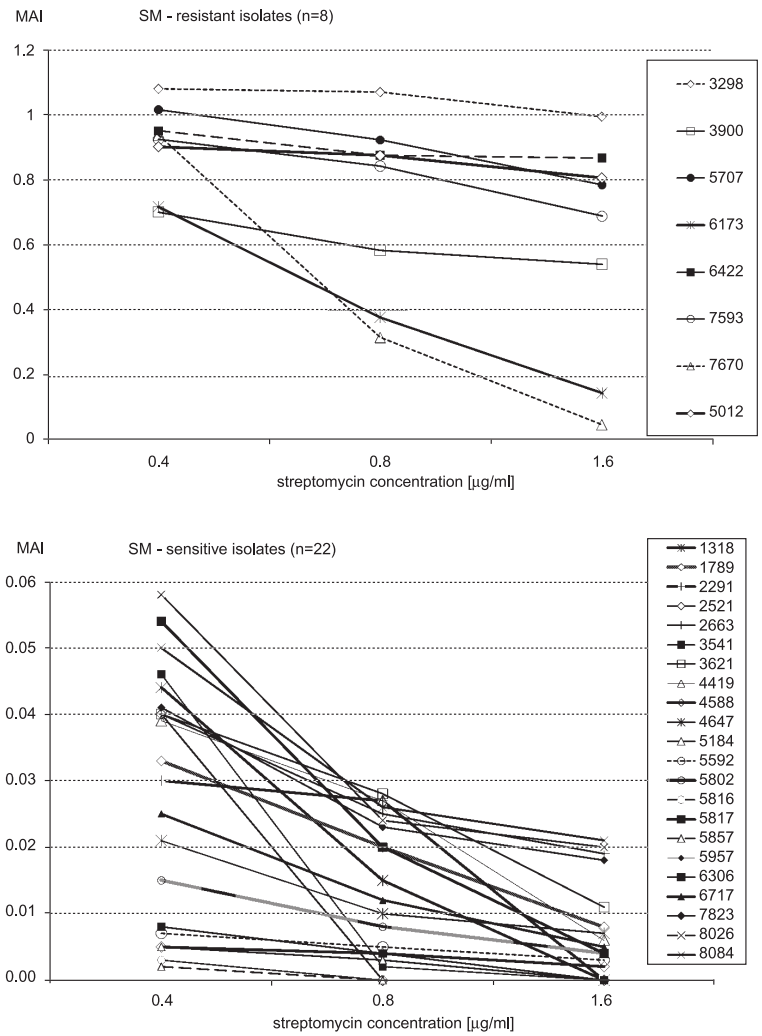

Fig. 4. Mycolic Acids Index (MAI) of SM-sensitive and SM-resistant isolates of *M.tuberculosis* cultured with different concentrations of the drug

Table 2. Comparison of drug resistance of *Mycobacterium tuberculosis* strains according to conventional tests performed in solid Lowenstein – Jensen medium (L-J) and liquid medium in MGIT system to evaluation of HPLC analysis of mycolic acid index (MAI)

Strain	INH			RMP			EMB			SM		
	L-J 0.2 µg/ml	MGIT 0.1 µg/ml	MAI 0.1 µg/ml	L-J 40.0 µg/ml	MGIT 1.0 µg/ml	MAI 1.0 µg/ml	L-J 2.0 µg/ml	MGIT 3.5 µg/ml	MAI 3.5 µg/ml	L-J 4.0 µg/ml	MGIT 0.8 µg/ml	MAI 0.8 µg/ml
1318/01	S	S	0	S	S	0	S	S	0.028	S	S	0.015
1789/02	S	S	0.006	S	S	0.008	S	S	0.023	S	S	0.020
2291/02	S	S	0	S	S	0	S	S	0.016	S	S	0.027
2521/02	S	S	0	S	S	0	S	S	0.017	S	S	0
2663/02	S	S	0.007	S	S	0.016	S	S	0.016	S	S	0.030
3541/02	S	S	0.008	S	S	0	S	S	0.024	S	S	0.003
3621/02	S	S	0.022	S	S	0	S	S	0.022	S	S	0.028
4419/03	S	S	0.005	S	S	0.012	S	S	0.023	S	S	0.027
4588/03	S	S	0	S	S	0	S	S	0.004	S	S	0.004
4647/03	S	S	0.004	S	S	0.009	S	S	0.007	S	S	0.010
5012/03	S	S	0.026	S	S	0.028	S	S	0.002	R	R	0.877
5184/03	S	S	0	S	S	0.007	S	S	0.002	S	S	0.003
5592/03	S	S	0.007	S	S	0.017	S	S	0.006	S	S	0.005
3298/04	R	R	0.914	R	R	0.918	S	S	0	R	R	1.071
3900/04	R	R	0.827	S	S	0.019	S	S	0.015	R	R	0.585
5707/04	S	S	0.021	S	S	0.031	S	S	0.002	R	R	0.924
5802/04	R	R	0.842	S	S	0.016	S	S	0.014	S	S	0.008
5816/04	S	S	0.019	S	S	0.025	S	S	0	S	S	0
5817/04	S	S	0.004	S	S	0.004	S	S	0.013	S	S	0.020
5857/04	S	S	0.015	S	S	0.012	S	S	0	S	S	0
5957/04	S	S	0.014	S	S	0.018	S	S	0	S	S	0.004
6173/04	S	S	0.012	S	S	0.006	S	S	0.015	R	R	0.379
6306/04	S	S	0.028	S	S	0.009	S	S	0.002	S	S	0.004
6422/04	S	S	0.006	S	S	0.011	S	S	0	R	R	0.877
6717/04	S	S	0.013	S	S	0.018	S	S	0	S	S	0.013
7593/04	S	S	0.021	S	S	0.014	S	S	0.002	R	R	0.844
7670/04	R	R	0.930	S	S	0	S	S	0.049	R	R	0.318
7823/04	S	5	0	R	S	0	S	S	0.019	S	S	0.023
8026/04	R	R	0.726	S	S	0.003	S	S	0.025	S	S	0.024
8084/04	R	R	0.878	S	S	0.012	S	S	0.013	S	S	0.026

all examined drug-sensitive and drug-resistant strains. The respective values of MAI at critical concentrations of the drugs and the results of susceptibility testing with conventional methods are compared in Table 2. The highest MAI values for all examined drug-susceptible strains tested at the critical concentration of a drug did not exceed 0.132, 0.147, 0.272 and 0.171 for INH, RMP, EMB and SM, respectively, and were significant lower than the lowest MAI values for the resistant strains: 3.167 for INH, 4.051 for RMP and 1.761 for SM. No *M.tbc* isolate was EMB resistant and only one revealed resistance to RMP. Generally, full agreement was obtained between the three methods of sensitivity testing: with the use of MAI evaluation, the MGIT system and the conventional proportion method. Only a single discrepancy was observed between the results obtained by the proportion method and those by the MGIT and HPLC analyses for 120 tests performed – it concerned one of the two

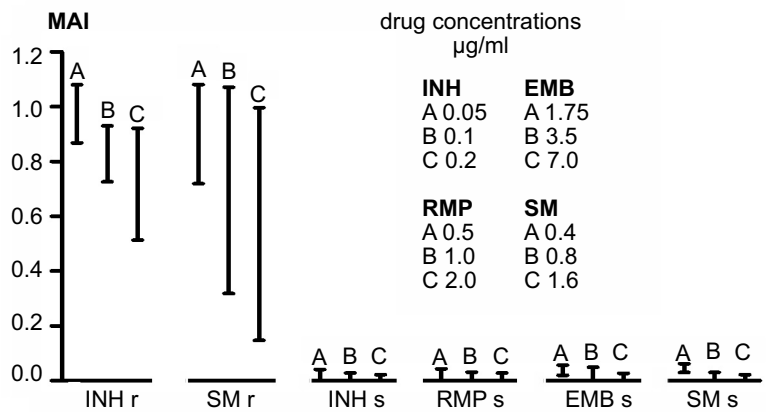

Fig. 5. Mycolic Acids Index (MAI) for the drug-resistant (r) and drug-susceptible (s) strains of *Mycobacterium tuberculosis*

Table 3. Drug resistance of *Mycobacterium tuberculosis* strains as evaluated by conventional tests and mycolic acid HPLC analysis

	INH		RMP		EMB		SM	
	strains resistant /n/	strains susceptible /n/	strains resistant /n/	strains susceptible /n/	strains resistant /n/	strains susceptible /n/	strains resistant /n/	strains susceptible /n/
HPLC analysis	6	24	1	29	0	30	8	22
Proportion method	6	24	2	28	0	30	8	22
MGIT system	6	24	1	29	0	30	8	22

isolates defined as RMP-resistant by the proportion method but classified as sensitive by the MGIT and mycolic acid analysis (Table 3).

4. Discussion

We evaluate here the usefulness of measurement of mycolic acids' content by HPLC for drug susceptibility testing in relation to two current and commonly used methods: the proportion method on solid (L-J) medium, and MGIT. The proportion method is still the most preferred one in many countries. Generally, in this method the number of colonies on drug-free medium is compared with the number of colonies cultured on drug-containing medium. A crucial limitation of the proportion method is time consuming for its performance (up to 6 weeks). An additional disadvantage stems from the clumping of bacilli in the seeded inoculum, which makes the number of growing colonies a poor representation of the actual number of viable bacterial cells: depending on the degree of clumping, a colony may form different number of cells. For this reason, the term CFU (colony forming units) is more appropriate. Because of clumping the proportion method introduces errors in susceptibility testing [23]. The second reference method in our study – MGIT is probably hardly affected by clumping of tubercle bacilli. Moreover, the growth of mycobacteria is better in liquid medium, the test is more sensitive and more rapid than that performed on L-J slants [21, 24]. Clumping was probably the reason of the discrepancy between the results of the proportion method and the MGIT system for the single isolate in our study.

The advantage of HPLC analysis of mycolic acid content in drug-susceptibility testing is that it measures absolute levels of components of the bacterial cell wall. The HPLC analysis of p-bromophenacyl bromide esters of mycolic acids developed by Butler et al. [16] is commonly accepted and used as a "gold standard" for differentiation of tubercle bacilli to the species level [17] but only a few authors have applied this technique for susceptibility testing. In 1997, preliminary studies by Garza-Gonzales et al. [25] documented a linear relationship between the logarithm of CFU per milliliter and TAMA, and showed that it was possible to detect growth inhibition of *M.tuberculosis* in presence of INH and SM by using HPLC. In 2001, Viader-Salvadó et al. [26] compared the susceptibility/resistance to INH and RIF by MAI (mycolic acid index) and the indirect-proportion method for a total of 200 clinical isolates of *M.tuberculosis*. Both the methods gave concurring results for 398 of the total of 400 tests (99.5%). They used coumarin as a fluorescent derivatizing agent for mycolic acids to increase sensitivity of detection. Recently, the fluorescent derivatives of mycolic acids were used in the susceptibility testing by HPLC by Parrish at al. [27]. The Bactec radiometric growth system [24] was the reference method in that study. At 72 h, the agreement of the HPLC method with the reference one ranged from 98.7 for RIF to 99.5% for INH, EMB, and PZA. However, using this method SM resistance was not detected in 15 of 22 resistant strains. The authors suggested

that it was due to different levels of SM resistance which is mediated by a number of factors, including mutations in *rpsL* (associated with high-level resistance) and *rrs* (associated with intermediate resistance) and altered membrane permeability of examined strains which contributes to resistance could produce a negative impact on the growth rate. We found that critical step of drug-susceptibility testing by quantitative evaluation of TAMA by HPLC technique, is the size of the inoculum, which determines the period of linear relationship between TAMA values and duration of culture. Besides dispersion and viability, also size of inoculum has a significant effect on the results of drug-susceptibility tests [28]. In our study the use of an inoculum acc. McF <0.5 and a 5-day culture enabled the clear distinction of MAI values between the SM-resistant and SM-sensitive strains, in spite of the significant differences in the level of the SM-resistance between the strains – the MAI values of the eight SM-resistant strains cultured with the drug at 1.6 μg /ml medium ranged between 0.05 and 1.0.

In the presented study, we evaluated reliability of the HPLC analysis of mycolic acids for susceptibility testing of *M.tuberculosis* to three first-line drugs and only one secondary drug. Further studies with more clinical isolates and extension to other drugs are needed but we believe the research carried out up to now allows the following conclusions to be drawn.

5. Conclusion

Comparison of mycolic acid contents in 5-day cultures in drug-containing and drug-free liquid medium by HPLC is a rapid and accurate method for testing of drug-sensitivity of tubercle bacilli. The method permits to save a lot of time because the commonly used conventional techniques used to evaluate drug effects on the number of CFU take about 4–6 weeks. However, the method has technical limitations in routine clinical laboratories and presently may be recommended mostly for laboratories which examine susceptibility of mycobacteria on new synthesized compounds.

Acknowledgment

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