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Determination of Drug Susceptibility of *Mycobacterium tuberculosis* through Mycolic Acid Analysis

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In the present work a rapid method to determine the susceptibility of *Mycobacterium tuberculosis* to isoniazid and streptomycin by determining levels of mycolic acids by high-performance liquid chromatography (HPLC) was developed. Mycobacterial growth kinetics in the presence and absence of antituberculosis drugs was characterized by evaluating the total area corresponding to mycolic acid peaks (TAMA). Results show a linear relationship between the logarithm of CFU per milliliter and TAMA and show that it is possible to detect growth inhibition of *M. tuberculosis* in the presence of isoniazid or streptomycin by using HPLC in 3 and 4 days, respectively.

High-performance liquid chromatography (HPLC) is at present used for the identification of mycobacteria through mycolic acid analysis (2–6, 8, 9, 12). Mycolic acids are high-molecular-weight, α-alkyl-β-hydroxy fatty acids of 70 to 90 carbon atoms (1). Standard techniques to determine the drug susceptibilities of *Mycobacterium tuberculosis* isolates require 3 weeks to complete (11); for this reason it is very important to develop a rapid drug susceptibility test to support optimal patient treatment. The need for such a test is more urgent now due to the reappearance of tuberculosis (TB) as a worldwide public health problem and above all by the appearance of multiple-drug-resistant strains. In the present work a rapid method to determine the susceptibility of *M. tuberculosis* to isoniazid and streptomycin by determining levels of mycolic acids was developed.

M. tuberculosis H37Ra (ATCC 25177) strain was inoculated onto a Lowenstein-Jensen slant and incubated for 4 weeks at 35 to 37°C. Growth was scraped from the slant and transferred to a tube (16 by 125 mm) containing 6 to 8 glass beads and 3 ml of 0.05% Tween in saline solution. The absorbance at 540 nm was adjusted to a corresponding McFarland 1 standard. This suspension is designated the mycobacterial work suspension (MWS).

For mycolic acid analysis standardization, a modification of the procedure described by Butler and Kilburn (3) was followed. A screw-cap tube (13 by 100 mm) with 1 ml of MWS was prepared, and 1.0 ml of saponification reagent (50% KOH in 75% methanol) was added. Whole cells were saponified at 121°C for 1 h. Mycolic acids were acidified with 6 N HCl and extracted three times with 1 ml of HPLC-grade CH_2Cl_2. Organic extracts were collected in a screw-cap tube (13 by 100 mm), evaporated to dryness with a nitrogen stream and heating at 60°C, and cooled at room temperature. Then, 0.1 ml of 0.2 M $KHCO_3$ was added and evaporated to dryness with a nitrogen stream. To this dried extract, 1 ml of HPLC-grade CH_2Cl_2 and 50 μl of *p*-bromophenacyl-8 reagent (Pierce Chemical Co., Rockford, Ill.) were added. The tube was perfectly sealed, heated at 85°C for 20 min, and cooled at room temperature, and 1 ml of 12 N HCl-methanol-water (1:2:1) was added. The tube was thoroughly mixed, and the organic phase was removed. The aqueous phase was extracted twice more with 0.5 ml of HPLC-grade CH_2Cl_2. Organic extracts were collected in a 2-ml Eppendorf tube and evaporated to dryness with a nitrogen stream and heating at 60°C. The dry residue was dissolved in 70 μl of cool HPLC-grade CH_2Cl_2, the mixture was centrifuged at 16,000 × *g* (14,000 rpm) in a microcentrifuge for 3 s, and 20 μl was injected immediately. Conditions and instrumentation for the analysis of mycolic acid *p*-bromophenacyl esters were as described previously (6). Peaks at retention times between 7 and 10 min were identified as mycolic acid levels and the total area corresponding to mycolic acid peaks (TAMA) were determined for each chromatogram.

Characteristic chromatograms of mycolic acids were obtained with the *M. tuberculosis* pattern as described by Butler et al. (3, 5). A mycolic acid analysis precision of 14% was obtained by evaluating the TAMA coefficient of variation of a sample analyzed four times.

M. tuberculosis growth kinetics was determined in the presence and absence of anti-TB drugs by evaluating TAMA by HPLC and by plate counts of CFU per milliliter to validate the growth kinetics determination by HPLC and to correlate TAMA with CFU per milliliter. All growth kinetics determinations were carried out in 42 sterile screw-cap tubes (24 by 100 mm) with 10 ml of Middlebrook 7H9. Tubes were inoculated with 10 μl of MWS and incubated at 35 to 37°C with constant agitation of 13 rpm in a Glas-Col agitator (Laboratory Rotator, Terre Haute, Ind.). Isoniazid (0.2 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) or streptomycin (2.0 μg/ml) (streptomycin sulfate; Sigma Chemical Co.) was used in growth kinetics determinations for cultures exposed to anti-TB drugs. Three tubes were removed from incubation at days 0, 1, 2, 3, 4, 5, 8, 11, 14, 17, 20, 23, 26, and 29, always at the same hour. The removed tubes were mixed thoroughly with a vortex for 15 min, and 100 μl of mycobacterial suspension was used to prepare dilutions of 1:10, 1:100, 1:1,000, and 1:10,000. One-tenth mil-

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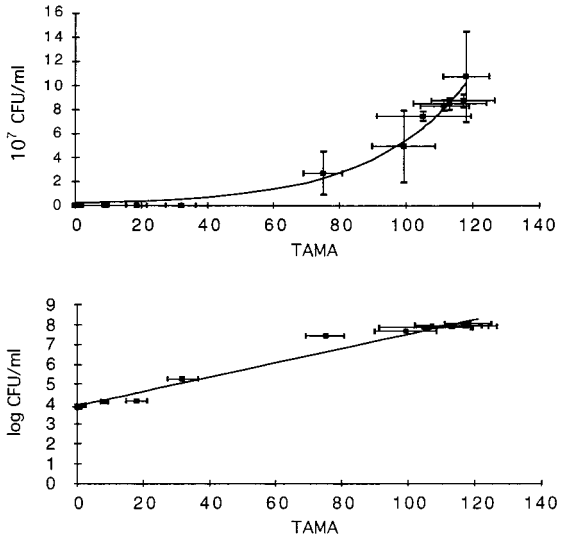

FIG. 1. Variation of CFU per milliliter versus TAMA (upper plot) and variation of the logarithm of CFU per milliliter versus TAMA (lower plot). Each point is the mean ± standard error of the mean ($n = 3$), and the curves represent the mathematical fit into the exponential equation CFU/ml = $1.6 \times 10^6 e^{0.35\ TAMA}$ ($r^2 = 0.95$; $P < 0.001$) (upper plot) and the linear equation log CFU/ml = 3.7×10^{-2} TAMA + 38 ($r^2 = 0.98$; $P < 0.001$) (lower plot).

liliter of each dilution was inoculated onto Middlebrook 7H10 agar plates and incubated at 35 to 37°C for 4 weeks for determinations of CFU per milliliter by the plate count method. To verify culture purity, 5 μl of mycobacterial suspension was used for staining by the Ziehl-Neelsen method (11). Tubes with the rest of the mycobacterial growth were frozen at −20°C until TAMA determination. TAMA determinations were as described above except that the volume was 10 ml and saponification and acidification reagents were increased 10-fold. All steps after the addition of reagents were performed as described above. Growth kinetics assays evaluated by HPLC were carried out three times, and the TAMA coefficients of variation obtained were less than 17% after the third incubation day.

Figure 1 (upper plot) shows the relationship of CFU per milliliter and TAMA for each day analyzed. The experimental data were fitted into an exponential curve ($r^2 = 0.95$; $P < 0.001$) by the nonlinear regression method. This relationship was verified by the linear relationship ($r^2 = 0.98$; $P < 0.001$) obtained in the semilogarithmic plot of CFU per milliliter versus TAMA (Fig. 1, lower plot), indicating that both parameters are directly proportional and that it is possible to calculate the CFU per milliliter of a mycobacterial suspension by using the TAMA value. This represents a decrease in the time necessary to analyze a sample by the plate count method, which requires a minimum of 3 weeks of incubation. The TAMA determination requires a minimum of 2 h.

Figure 2 shows a superimposed plot of the logarithm of CFU per milliliter versus time and TAMA versus time. The experimental data were fitted into sigmoid equations ($r^2 = 0.97$ and 1.00, respectively; $P < 0.001$). On the logarithm of CFU per milliliter versus time plot, the lag, exponential, and stationary phases of mycobacterial growth are seen. In the 0-to-6-day portion of this plot a slight increase of CFU per milliliter with respect to the initial value was observed (10.2%) and corresponds to the lag phase of growth kinetics. However, in the TAMA versus time plot a greater increase of TAMA with respect to the initial value was observed (20,800%), indicating

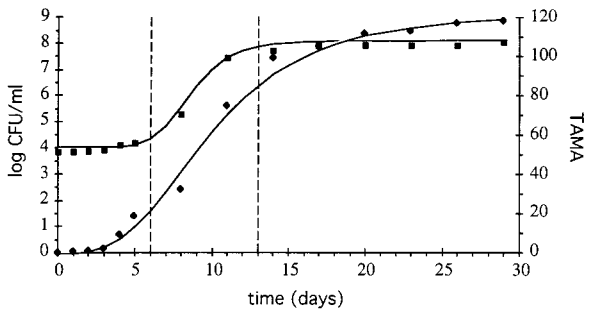

FIG. 2. Variation of log CFU per milliliter versus time (■) and TAMA versus time (◆) in the kinetics assays performed in the absence of anti-TB drugs. Each point is the mean of three assays, and the curves represent the mathematical fit into sigmoid equations log CFU/ml = $[4.1t^{6.8}/(t^{6.8} + 2.3 \times 10^6)] + 4.0$ ($r^2 = 0.97$; $P < 0.001$) and TAMA = $124.9t^3/(t^3 + 1{,}000)$ ($r^2 = 1.0$; $P < 0.001$), respectively. Vertical dashed lines indicate growth phase limits.

that although cellular proliferation was minimal, mycolic acids were synthesized. From day 6 to 13 a linear relationship with similar slopes in both plots was observed (0.09 and 0.11 for the CFU per milliliter and TAMA plots, respectively) and corresponds to the exponential phase. This relationship indicates that increments of the logarithm of CFU per milliliter and TAMA per unit time are constants and are similar; that is, if a first-order reaction is assumed, then the rate constants are similar. From day 13 onward a small increase of TAMA (18.9%) was observed, although CFU per milliliter remained constant; this corresponds to the stationary phase.

The upper plot in Fig. 3 shows the changes of the logarithm of CFU per milliliter versus time and TAMA versus time for cultures exposed to isoniazid. From day 0 to 4 the logarithm of CFU per milliliter versus time decreased and thereafter re-

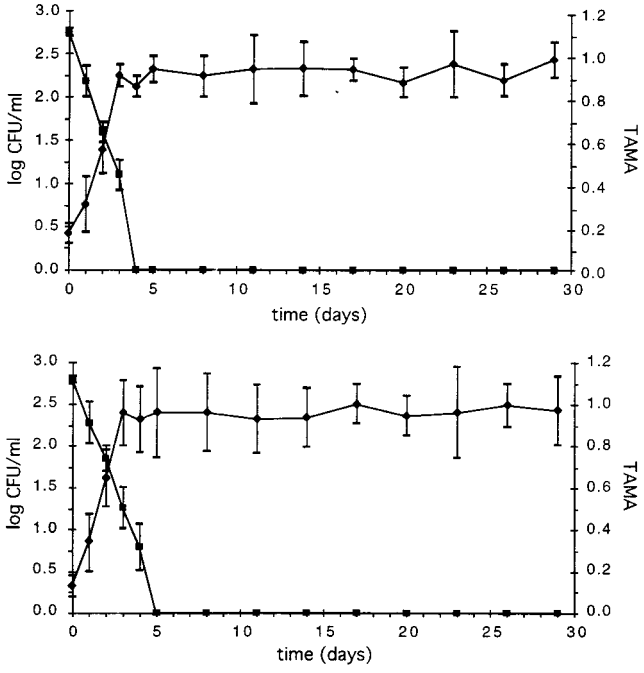

FIG. 3. Variation of the logarithm of CFU per milliliter versus time (■) and TAMA versus time (◆) in the growth kinetics assays performed in the presence of isoniazid (upper plot) or streptomycin (lower plot). Each point is the mean ± standard error of the mean ($n = 3$).

mained constant. On the other hand, from day 0 to 3 TAMA increased and thereafter remained constant. A Student's *t* test assuming equal variances was used to compare the means of TAMA obtained in the presence and absence of isoniazid every day; significant differences ($P < 0.01$) were obtained from day 3 onward.

The lower plot in Fig. 3 shows the changes of the logarithm of CFU per milliliter versus time and TAMA versus time for cultures exposed to streptomycin. From day 0 to 5 the logarithm of CFU per milliliter versus time decreased and thereafter remained constant. On the other hand, TAMA increased from day 0 to 3 and thereafter remained constant. As in the case of growth kinetics in the presence of isoniazid, a Student's *t* test was done and significant differences ($P < 0.01$) for TAMA were obtained from day 4 onward.

Both statistically significant differences indicate that it is possible to detect *M. tuberculosis* growth inhibition in the presence of isoniazid or streptomycin by using HPLC in 3 or 4 days, respectively, while the traditional indirect proportion culture method requires 3 weeks of incubation to obtain susceptibility results. Thus, it is possible to use mycolic acid analysis as a rapid method of testing the drug susceptibility of *M. tuberculosis* isolates, and since this analysis is presently used in assays for the identification and differentiation of *Mycobacterium* species, it can confirm a TB patient diagnosis.

On the basis of the results obtained, we suggest the use of HPLC analysis of mycolic acids to determine the susceptibility of *M. tuberculosis* isolates to anti-TB drugs. We suggest that this be done by evaluating the initial TAMA ($TAMA_0$) and the TAMA at the 5th incubation day ($TAMA_5$) in the presence of the anti-TB drug and calculating $100(TAMA_5 - TAMA_0)/TAMA_0$ to obtain the percentage of TAMA increase. These results must always be compared with a nondrug control. As mycobacterial identification by HPLC can be done at present by using fluorescence detection (10) and since this type of detection is more sensitive than UV detection, its use in susceptibility tests will have the advantage of reducing the sample volume from 10 to 1 ml.

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