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A rapid method of diagnosing pulmonary tuberculosis using stir bar sorptive extraction–thermal desorption–gas chromatography–mass spectrometry

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Abstract

A fast method for detection of tuberculostearic acid (TBSA) in sputum samples is described. The samples, obtained from patients with known or suspected pulmonary tuberculosis, were decontaminated and concentrated before being analyzed by stir bar sorptive extraction–thermal desorption–gas chromatography–mass spectrometry (SBSE–TD–GC–MS). Prior to extraction, the mycobacterial lipids were hydrolyzed and then derivatized with ethyl chloroformate to increase the sorption of the compounds by the polydimethylsiloxane (PDMS) stir bar coating. The limit of detection (LOD) is 0.2 ng ml^{-1}. Four sputum samples that were classified by direct microscopy as smear-positive or negative were analyzed by GC–MS. TBSA was detected at concentrations ranging from 0.47 to 2.3 ng ml^{-1}. The method is sufficiently sensitive to detect TBSA directly in clinical samples without the need to culture the organisms.
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1. Introduction

There is an increased demand for the rapid detection and identification of *Mycobacterium tuberculosis* due to the re-emergence of diseases associated with this organism. *M. tuberculosis* indeed is frequently associated with the human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS), which has led to the increased prevalence of pulmonary tuberculosis (TB) globally, but particularly in sub-Saharan Africa [1].

Traditional methods used for the identification of mycobacteria include direct microscopy and culturing, which form the basis of present-day laboratory diagnosis of TB. Culturing is often the only decisive test of mycobacterial disease, but since the organisms are slow-growing it may take up to 8 weeks to obtain results [2]. More recently, new diagnostic methods such as the BACTEC radiometric system and the polymerase chain reaction (PCR) have decreased the time required to diagnose tuberculous infections (i.e. 10–14 days) [3]. However, most laboratories in developing countries are ill equipped to successfully utilize these techniques because of inadequate facilities and lack of funding [4].

Gas chromatography was first used as a tool in diagnosing TB in the late 1970s [5]. However, the use of chromatographic methods for the identification of mycobacteria has been restricted to larger reference and research laboratories [6]. Reasons include the cost and maintenance of equipment, the time required to prepare samples, and the fact that most gas chromatographic methods require a culture-step prior to the analysis of clinical samples [7–10].

Stir bar sorptive extraction (SBSE) was developed to facilitate the direct extraction of organic trace compounds from aqueous samples [11]. Rapid, solventless extraction is achieved by using a stir bar coated with a layer of polydimethylsiloxane (PDMS). Target analytes are enriched in

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a reproducible way by a partitioning mechanism based on the water–octanol distribution constants ($K_{o/w}$) of the solutes [12] when extrinsic factors such as time, pH, temperature, and magnetic stirring speed are held constant. For polar solutes, the introduction of a derivatization-step prior to extraction enhances their sorption by the stir bar coating. Finally, the stir bar is desorbed using a thermal desorption (TD) device and analyzed by gas chromatography–mass spectrometry (GC–MS).

The aim of this investigation was to demonstrate the direct measurement of a chemical marker of tuberculosis, namely tuberculostearic acid (TBSA), in sputum samples using SBSE–thermal desorption–GC–MS.

2. Experimental

2.1. Chemicals and materials

Ethyl chloroformate, pyridine, sodium hydroxide, 17-methyloctadecanoic acid (MODA, internal standard) and 10-methyloctadecanoic acid (tuberculostearic acid, TBSA) were purchased from Sigma–Aldrich (Johannesburg, South Africa). Ethanol and chloroform were obtained from Merck (Darmstadt, Germany). Hydrochloric acid was supplied by Acros (Geel, Belgium). The sonicator was a Branson model 3510 obtained from LASEC (Cape Town, South Africa). The 15 ml screw cap vials were from Supelco (Sigma–Aldrich, Johannesburg, South Africa). Stir bars (10 mm × 0.5 mm) with a 24 μl PDMS coating were purchased from Gerstel GmbH (Müllheim a/d Ruhr, Germany).

2.2. Sputum samples

Sputum samples were obtained from routine clinical specimens that were sent to the National Health Laboratory Service (NHLS, Cape Town, South Africa). Approximately 5–10 ml of a first-morning sputum specimen was collected from patients with known or suspected pulmonary tuberculosis. Each sample was classified as 1+, 2+, or 3+ positive based on the results obtained by direct microscopy. One sample was analyzed in each category, including a sample that was smear-negative. Five samples, obtained from patients with nonmycobacterial pneumonia that had no previous history of pulmonary tuberculosis, were used as controls. All samples were concentrated and decontaminated according to a standard procedure used by the NHLS [13]. The procedure was carried out with slight modification to the approved WHO method. Briefly, an equal volume of Sputagest was added to each sample and placed in an orbital shaker/incubator at 37 °C for 10 min at 330 rpm. One ml aliquots were removed and reserved as spare samples. The remainder of the specimens was decontaminated with equal volumes of 1 M sodium hydroxide and 0.1 M sodium citrate, such that the final volume was twice that of the sample volume. The samples were again placed in the orbital shaker/incubator for 20 min using the same settings. Two volumes of phosphate buffer (pH 6.8) were added and the samples were centrifuged at 3000 rpm for 20 min. The supernatant aqueous phase was removed and the sample pellets were re-suspended in 1.5 ml phosphate buffered saline and mixed gently. A 0.5 ml aliquot was used for culturing and direct microscopy while the remainder was centrifuged, the supernatant removed and the residue re-suspended in 0.5 ml phosphate buffered saline for GC–MS analysis. The samples were autoclaved at 180 °C for 20 min before being dispatched to the Chemistry Department at Stellenbosch University. All samples were stored at −20 °C until analyzed.

2.3. Sample preparation and SBSE procedure

The samples were transferred to 2 ml autosampler vials and 120 μl of concentrated hydrochloric acid (36%) was added to hydrolyze the bacterial lipids. The vials were capped and heated at 80 °C for 30 min. After cooling to room temperature, 0.5 ml of a 0.5 M sodium hydroxide solution was added to the vial and vortexed gently. The content was transferred to 15 ml glass vials with Teflon-lined screw caps and spiked with a 10 μl solution of the internal standard, namely 17-methyloctadecanoic acid, containing 260 μg/l in ethyl acetate. This corresponds to 2.6 ng in the sample vial. The fatty acids were derivatized using ethyl chloroformate according to a previously described procedure [12]: 1.04 ml of a mixture of ethanol and pyridine (5.5:1) were added to the vial followed by 80 μl of ethyl chloroformate (ECF) that was added under sonication in a fume hood. The open vial was sonicated for 15 min before placing a conditioned stir bar in the sample. The stir bars were conditioned at 280 °C under a nitrogen flow in a separate GC oven prior to SBSE. The samples were stirred at 1000 rpm for 30 min and extraction took place at 40 °C. After extraction of the analytes, the stir bar was removed from the sample, washed with distilled water and briefly dried with lint-free tissue paper to remove residual water droplets. The stir bars were the placed in a glass TDS tube for thermal desorption and analysis by GC–MS.

2.4. Instrumental conditions

2.4.1. Thermal desorption–capillary GC–selected ion monitoring–MS

The stir bars were thermally desorbed using a TDS-2 thermal desorption unit (Gerstel) operated in the solvent-venting mode. Thermal desorption was accomplished by increasing the temperature from 50 to 150 °C (held for 1 min) at 60 °C min^{-1} using a helium flow-rate of 60 ml min^{-1}. After 2 min, the TDS split-valve was closed followed by a final temperature increase to 300 °C (held for 10 min) at 60 °C min^{-1}. The desorbed compounds were transferred to a programmable temperature vaporization (PTV) inlet (CIS 4, Gerstel) through a heated transfer line (325 °C). Cryofocussing of the compounds took place at 40 °C in a baffled liner using liquid nitrogen. Sample injection was per-

formed in the splitless mode by programming the CIS 4 from 40 to 300 °C (held for 5 min) at 12 °C s^{-1}. The split-valve was opened after 2.5 min. GC–MS analyses were performed on a Hewlett-Packard 5890 gas chromatograph interfaced with a model 5972 mass spectrometric detector (MSD). Chromatographic separations were carried out on a HP-5MS fused-silica capillary column (Agilent Technologies; 30 m × 0.25 mm i.d. × 0.5 μm film thickness) using helium as the carrier gas at a flow rate of 1.4 ml min^{-1}. The oven temperature was programmed from 90 °C (held for 6 min) to 300 °C at 15 °C min^{-1}. The final temperature was held for 10 min. The transfer line temperature was held at 280 °C. The MSD was operated in both the scan and the selected ion monitoring (SIM) modes with an electron ionization voltage of 70 V. For SIM, two ions characteristic for the fatty acid-ethyl esters (*m/z* 88, 101) were monitored at 100 m/s for both ions.

2.5. Figures of merit

The linearity of the method was evaluated by setting up an internal standard calibration curve in control sputum samples that were obtained from patients with nonmycobacterial pulmonary infections. Standard stock solutions were prepared in chloroform at a final concentration of 0.7 μg ml^{-1} for TBSA and 1 μg ml^{-1} for the internal standard MODA. The samples were spiked with different concentrations of TBSA at 0.4, 0.7, 1.5 and 2.2 ng ml^{-1} to construct the calibration curve.

3. Results and discussion

Preliminary experiments performed on aqueous solutions of TBSA and the internal standard MODA, showed that extraction recoveries of ca. 90% were obtained using the conditions described in the experimental part. Moreover, because of the structural similarity between TBSA and the internal standard MODA, no differences in recovery between both solutes were noted. Calibration was performed in control sputum samples to determine the effect of the sample matrix on the limit of detection of TBSA. A four point calibration curve using internal standard calibration (0.4–2.2 ng ml^{-1}) showed good linearity over this range with a correlation coefficient of 0.9965. The LOD for TBSA was calculated at a signal-to-noise level of 3 and was determined at 0.2 ng ml^{-1} for the control sputum samples. Spiked control sputum samples at 1.5 ng ml^{-1} were analyzed three times and the R.S.D. (%) was 4.8.

Five sputum samples containing different bacterial loads were analyzed by SBSE–TD–GC–MS, after the decontamination/concentration procedure was carried out by the NHLS. One sample was from a patient being treated for pulmonary infection caused by *Streptococcus pneumoniae* (sample A) while the other samples were classified by direct microscopy as 1+ (sample B), 2+ (sample C), 3+ (sample D) and one negative for the mycobacteria (sample E).

In sample A no TBSA was detected by SBSE–TD–GC–MS. Sample B was classified as 1+ positive by direct microscopy. Smears stained for acid-fast bacilli (AFB) provide important preliminary information in the diagnosis of TB. It is also the least expensive method used in the detection of the mycobacteria, but the technique is known to lack specificity, which ranges from 25 to 80% [14]. No culture results were available for sample B but no TBSA was detected by GC–MS. The result obtained is therefore inconclusive because of the outstanding culture results and the fact that no other clinical information with regard to the patient's condition was provided.

Sample C was categorized as 2+ positive by direct microscopy and a clinical diagnosis of pulmonary TB was previously documented. At the time of the study, the patient was being re-treated for a previously acquired TB infection. The amount of TBSA detected by GC–MS was 0.47 ng ml^{-1} (Fig. 1, sample C). No information with regard to the type of medication used or the duration of treatment was available. The result was accepted as a 'true positive' because a diagnosis of pulmonary TB had previously been established and the results obtained by direct microscopy and GC–MS were both positive for the presence of the mycobacteria. Sample D was obtained from a patient diagnosed with disseminated tuberculosis. This is a severe form of the disease, which spreads to other parts of the body. The sample was classified as 3+ positive by direct microscopy and the microbial culture result was also positive. The concentration of TBSA determined by GC–MS was 2.3 ng ml^{-1} (Fig. 1, sample D). This sample is definitely a 'true positive' because all three tests performed were positive for the mycobacteria. Note that the two main peaks eluting before TBSA namely hexadecanoic and octadecanoic acid are strongly fluctuating. The reason for this phenomenon is unclear, although it may be attributed to the clinical course of the disease that is still not well understood.

Table 1
Comparison of data for GC–MS, direct microscopy and culturing

Number	Diagnosis	Direct microscopy	Mycobacterial culture	GC–MS (ng ml^{-1})
A	Sample (*Streptococcus pneumoniae*)	np	np	nd
B	New case (diagnosis to be confirmed)	1+ positive	np	nd
C	Pulmonary tuberculosis (retreatment)	2+ positive	−	0.472
D	Disseminated tuberculosis	3+ positive	+	2.27
E	Pulmonary tuberculosis (?)	Negative	−	nd

np: not performed; nd: not detected.

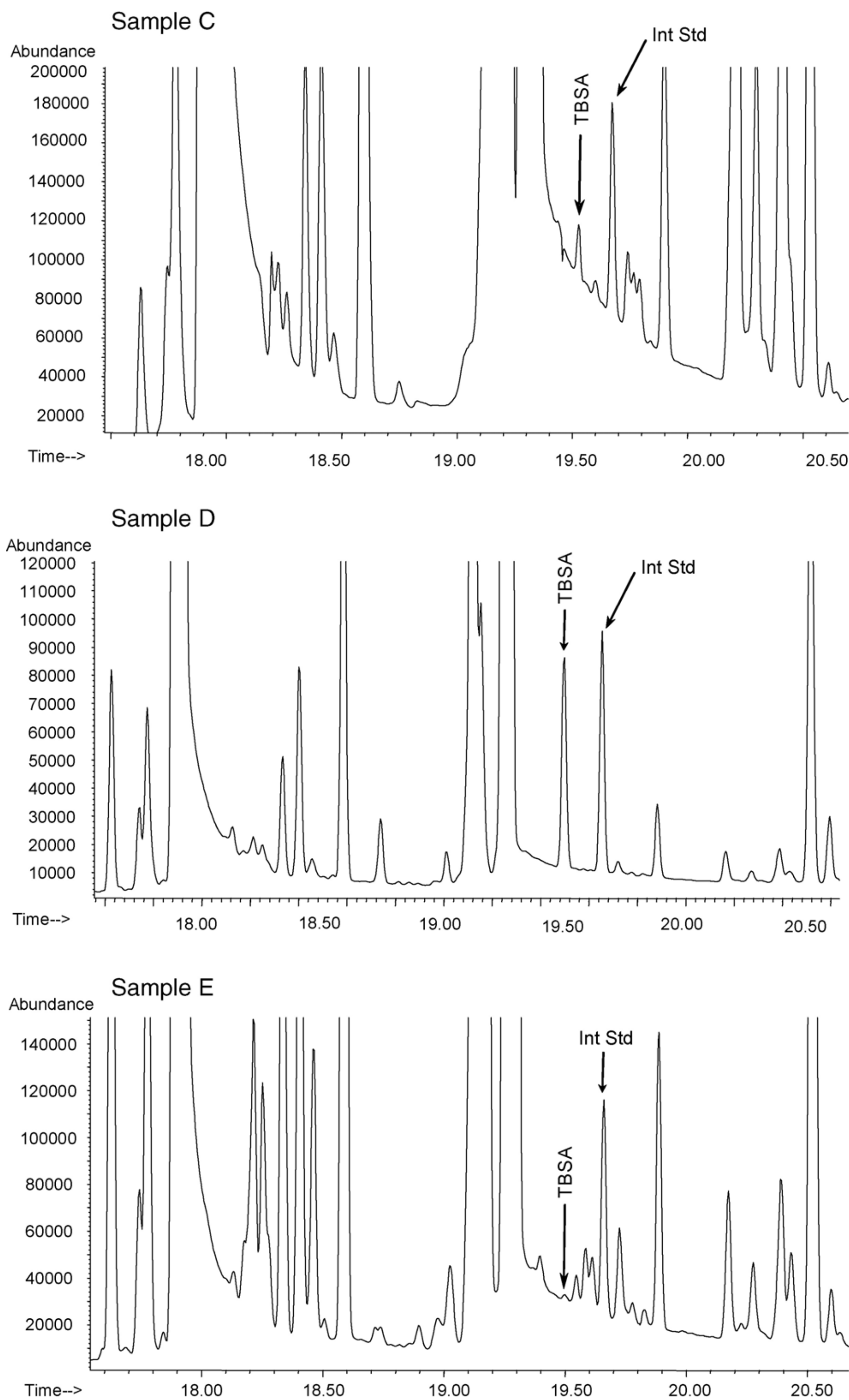

Fig. 1. SIM chromatograms (m/z 88,101) of sputum samples obtained by SBSE–TD–GC–MS. Sample C: 2+ positive, TBSA detected at 0.47 ng ml^{-1} (pulmonary TB); sample D: 3+ positive, TBSA detected at 2.3 ng ml^{-1} (disseminated TB); sample E: smear negative, culture negative, no TBSA detected by GC–MS.

For sample E, a diagnosis of pulmonary TB was documented following clinical assessment of the patient's condition. However, the AFB smear result was negative and no TBSA was detected by GC–MS (Fig. 1, sample E). Also, the culture result obtained from the MGIT system was still negative after a 6-week incubation period. The results obtained for direct microscopy, culturing and GC–MS are summarized in Table 1.

From the results obtained, it is clear that the GC–MS data follow the same trend as the conclusions derived from the direct microscopy and mycobacterial culture tests. However, more samples have to be analyzed to be able to determine the diagnostic sensitivity and specificity of the method. The results should also be compared to the clinical diagnosis given to each patient as well as the results obtained by standard culturing techniques to establish the positive and negative predictive values of the method. The type and duration of drug treatment should be considered and how it affects the detectable levels of TBSA. There is a significant advantage in being able to quantify the amount of TBSA in sputum samples. It may be used for example, to determine the progression of treatment and to establish whether a correlation exists between the concentration of TBSA and the extent of the disease. Moreover, the rapidity with which the method can be performed significantly reduces the time required to obtain clinically useful results without the need to culture the samples prior to analysis.

The use of TBSA alone for the diagnosis of pulmonary TB has been criticized in the past. TBSA is regarded as not specific enough due to its presence in other organisms such as the Actinomycetes, Nocardia and Rhodococcus species. However, the incidence of infections caused by these organisms as opposed to the mycobacteria should be considered. A study by Jones et al. [15] estimated that one case of pulmonary Nocardial infection occurred to 90 cases of pulmonary TB in a population of HIV infected patients. On the other hand, it would also be possible to increase the specificity of the TBSA method by identifying other markers that are specific for *M. tuberculosis*. A recent report by Alugupalli et al. [16] showed that certain 3-hydroxy fatty acids may be used to distinguish *M. tuberculosis* from other organisms. The interesting possibility that these compounds also provide a measure of the virulence of the mycobacteria deserves further investigation.

4. Conclusion

A rapid technique for the detection of TBSA in sputum samples was developed. Traditional decontamination and concentration methods were combined with SBSE–TD–GC–MS for the detection of TBSA at trace levels in clinical samples. The technique was sufficiently sensitive to detect TBSA without the need to culture the samples, thereby reducing the time required to obtain results. The future application of the method in the routine identification of the mycobacteria should be established by comparing the diagnostic accuracy of the technique against standard biochemical tests used to detect pulmonary tuberculosis.

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